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CONCENTRATION OF CORTICOTROPHIN BY PERCOLATION¹

M. SAFFRAN, B. MUHLSTOCK, AND BRIGITTE U. CAPLAN

Abstract

Percolation of commercial anterior pituitary powder with decreasing concentrations of ethanol (95 → 70%) and increasing concentrations of acetic acid (0 → 0.5 *M*) yielded a peak of corticotrophin activity with 70% ethanol containing 0.5 *M* acetic acid. The material assayed at about 14 units/mg peptide. Further purification by curtain electrophoresis yielded a preparation with 21 units/mg peptide.

In 1959, Bates, Garrison, and Howard (1) described a method for the extraction of thyrotrophin from pituitary and plasma powders by percolation with ethanol and aqueous salt solutions. By a very simple procedure the thyrotrophin was concentrated about 30 times. A modification of the Bates procedure has been used to concentrate corticotrophin from anterior pituitary powder, yielding material with a potency of about 14 units per mg in one step, which represents a concentration of about 80 times over the starting material.

Methods and Results

Twenty grams of an acetone powder of hog anterior pituitary tissue (lot CP 1960), assaying approximately 175 I.U. corticotrophin per gram, was mixed with an equal weight of Hyflo Super-Cel (Johns-Manville) filter aid. The mixture was passed through a 25-mesh sieve and then slurried with 200 ml 95% ethanol. The slurry was transferred to a 10 cm diameter Buchner funnel, precoated with a thin layer of Hyflo Super-Cel, also applied as a slurry in 95% ethanol on a circle of Whatman No. 41H filter paper. The filtrate was caught in a succession of test tubes of 50 ml capacity. The beaker that contained the slurry of pituitary powder was washed with 100 ml 95% ethanol and the washing was added to the Buchner funnel. Of the 300 ml of ethanol added, 165 ml was recovered in the filtrates; the retention volume of the filter

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cake was therefore 135 ml. The filter cake was percolated with ethanol with increasing amounts of water and of acetic acid in the concentrations and volumes listed in Table I. The average volume of each fraction was 40 ml. The filter cake swelled during the percolation, increasing the retention volume somewhat and slowing the rate of percolation. In the later fractions the rate of percolation was speeded up by the application of mild vacuum.

TABLE I
Percolation of anterior pituitary powder with ethanolic solutions

Fraction No. (inclusive)	Ethanol (%)	Water (%)	Acetic acid (M)	Total volume added (ml)	Volume recovered (ml)
1-9	95	5	0	465	323
10-16	90	10	0	300	286
17-23	85	15	0	200	285
24-30	80	20	0	300	287
31-37	75	25	0	300	277
38-44	70	30	0	300	287
45-51	70	30	0.01	300	279
52-58	70	30	0.05	300	287
59-65	70	30	0.1	300	278
66-72	70	30	0.2	300	279
73-85	70	30	0.5	600	523
86	0	100	0.5	600	Not measured

The fractions were concentrated to a volume of about 1 ml in a stream of filtered air at 50-55° in a vibrating Evapo-mix (Laboratory Glass and Instrument Corporation, New York). The concentrates were transferred, with washing, to test tubes of 7 ml capacity and were taken to dryness in a vacuum desiccator over NaOH pellets. The residues were dissolved in 3 ml of 0.5% aqueous acetic acid. Aliquots of these solutions were taken for ninhydrin reaction (slightly modified from (2)), for the Folin-Lowry method for peptides (3), and for biological screening. The results are plotted in Fig. 1. The material in every second fraction was tested at doses of 0, 0.1, 1.0, and 10 μ g of peptide on surviving rat adrenal tissue, according to the method of Saffran and Schally (4). In Fig. 1 the biological activity is expressed as the sum of the stimulation of adrenocorticoid production over the control by the three doses tested. The complete data are depicted in Fig. 2.

Significant biological activity appeared only in the tubes 76-86, represented by a Lowry-peptide peak and a small peak in the ninhydrin curve. The Lowry-peptide peak represents about 18% of the total peptide in all the fractions. Some of the fractions were assayed quantitatively against the U.S.P. standard (4). The assays showed that fractions 74-77 contained the most active corticotrophin, 8-14 units per mg (Fig. 3).

The corticotrophin is evidently soluble only in acidulated ethanol; the activity began to emerge, in detectable amounts, with 0.1 M acetic acid in 70% ethanol, and the major peak, with 0.5 M acetic acid.

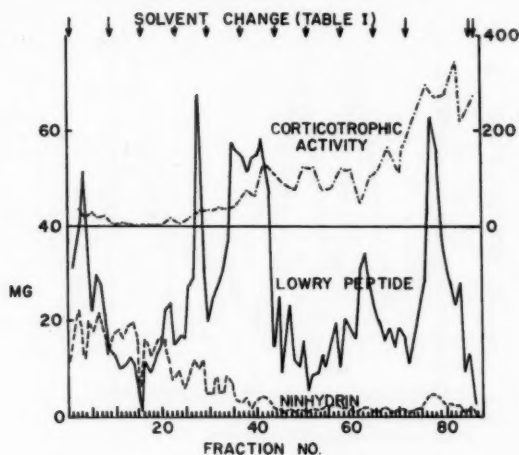


FIG. 1. Ninhydrin, peptide, and corticotrophic content of fractions obtained in the percolation of anterior pituitary powder. The corticotrophic activity on the right-hand side of the diagram is expressed in arbitrary units, composed of the sum of the increase in the formation of corticosteroids (measured as $[(O.D._{240} - O.D._{280}) / (\text{adrenal weight})] \times 10$) by three doses, 0.1, 1.0, and 10 μg , of each fraction.

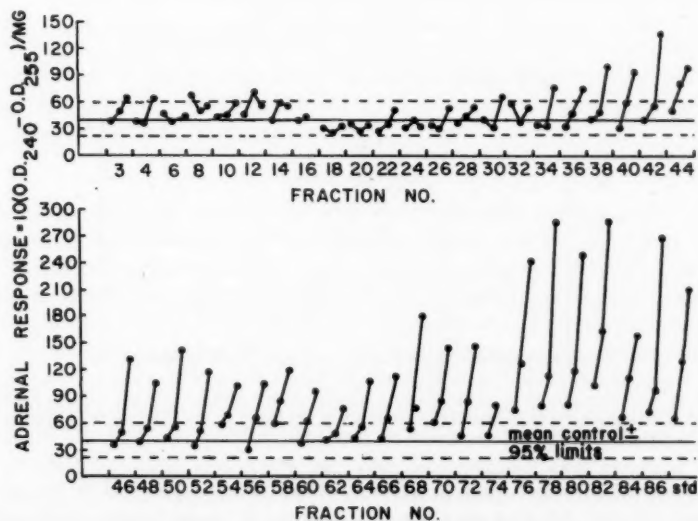


FIG. 2. The complete data of the screening for corticotrophic activity of every second fraction. Each fraction was tested at doses of 0.1, 1.0, and 10 μg . A typical response to 1, 10, and 100 milliunits of U.S.P. standard corticotrophin is in the lower right-hand corner of the diagram. The mean unstimulated (no added corticotrophin) control and its 95% fiducial limits are indicated by the solid and two dashed lines.

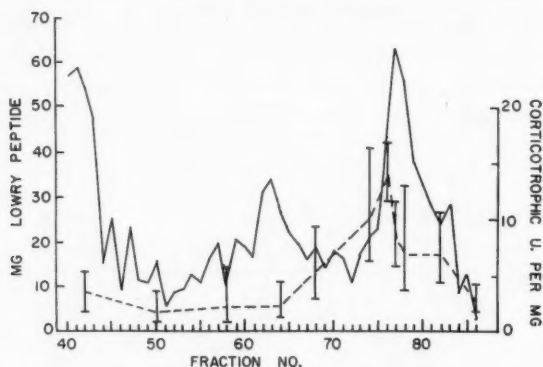


FIG. 3. Potency of corticotrophin in some fractions of the percolation. The activity is expressed in units per milligram of peptide and the potencies are accompanied by the 95% limits of the assays, shown as horizontal lines joined by a vertical bar.

The solution in fraction No. 77 was further subjected to electrophoresis in a Beckman/Spinco continuous flow electrophoresis apparatus, with 3% acetic acid (approx. 0.5 *M*) as electrolyte at 750 volts over about 21 hours.

The fractions were concentrated in the Evapo-mix, as above, but this time the residues were taken up in only 0.5 ml 0.5% acetic acid. Aliquots of these solutions were examined for Lowry peptides (Fig. 4) and the peak fractions

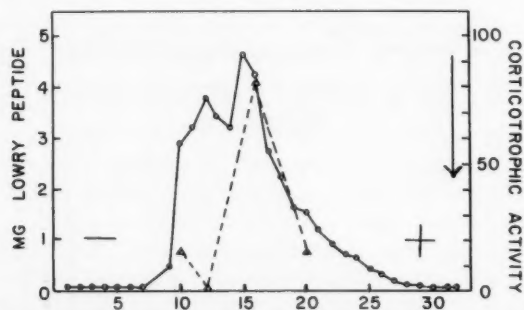


FIG. 4. Continuous flow paper electrophoresis of percolation sample No. 77. Corticotrophic activity (---) is expressed in arbitrary units (see Fig. 1). The point of application of the sample to the paper is indicated by the arrow.

were tested for corticotrophin activity at 0.01, 0.1, and 1 μ g. Only the peak in fractions 15 and 16 had significant activity. A quantitative assay of the material in fraction 16 showed a potency of 21 units per mg, with 95% limits of 10.4 and 43.2.

The reproducibility of the percolation step is illustrated in Fig. 5, in which a 200-g batch of hog posterior pituitary powder, contaminated with anterior

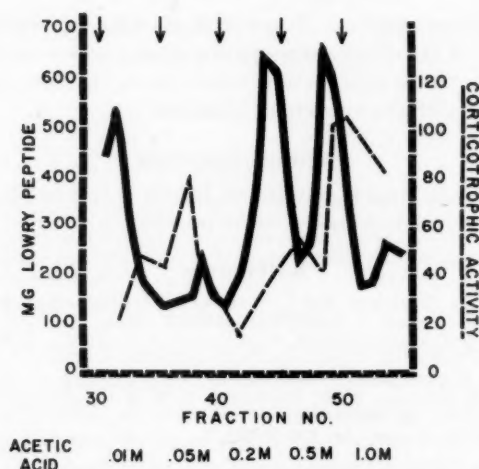


FIG. 5. Percolation of 200 g of posterior pituitary powder of the hog, contaminated with anterior lobe, with 70% ethanol containing acetic acid. The fractions obtained with unacidified ethanol are not shown. Fraction volume, about 500 ml. The corticotrophic activity is expressed as in Fig. 1. The arrows at the top of the figure indicate change of percolating solvent. Lowry peptide, solid thick line. Corticotrophic activity, broken thin line.

lobe, was used. In spite of differences in the scale of the experiment and in the composition and relative volume of the percolating solvents, the pattern is similar. Detectable corticotrophic activity appeared with more dilute acetic acid, but the major peak emerged with 0.5 M acetic acid.

Discussion

The combination of percolation and electrophoresis yielded corticotrophin of approximately the same range of potency as some of the "pure" preparations of corticotrophin, but not of the most potent β -corticotrophin, of Bell (5, 6). However, these two procedures are relatively mild and easily carried out, resulting in a 120-fold increase in potency over the starting material. Now that the activity has been located in the percolation and electrophoresis, the tedious testing of each fraction need not be repeated. We have ample evidence from similar experiments with brain and posterior pituitary powders (M. Saffran, B. U. Caplan, S. Mishkin, and B. Muhlstock, unpublished) that the two procedures yield extremely reproducible results. The product with a potency of 21 units/mg can serve as starting material for further purification by other techniques, such as ion exchange chromatography.

The screening procedure for corticotrophic activity consisted of a simple dose-response curve (Fig. 2), which selects the active fractions as those producing responses greater than the upper 95% limit of the controls. A guide to the relative activity of the fractions is furnished by the lowest dose which

produces a significant response. For ease in plotting the results of the screening test, the sum of the stimulations by the three doses was used (Figs. 1 and 5). The screening test is only an approximation, which was supplemented by quantitative assays of the important fractions.

Acknowledgments

We are indebted to Messrs. Antoft and Jensen of Nordic Biochemicals Ltd., Montreal, for generous supplies of tissue powders.

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ESTRADIOL-17 β IN THE EGGS OF THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*¹

ROBERT D. LISK²

Abstract

Eggs of the American lobster, *Homarus americanus*, were examined for estrogenic materials. Analysis by the Astwood 6-hour assay indicated 0.26 to 0.32 μ g of estrogenic activity per kilogram of eggs. Analysis by countercurrent distribution, paper chromatography, and bio-assay strongly suggests that the biologically active material is estradiol-17 β .

Introduction

Extracts which produce estrogenic reactions in mammals have been prepared from invertebrate material representing several phyla (1) but in most cases the chemical identity of these substances remains unknown. Hisaw and his associates at Harvard have, over the past number of years, sought to obtain positive identification of estrogenic materials found in the lower vertebrates and invertebrates. Using the methods commonly employed for obtaining estrogenic hormones from mammalian material, these workers have identified, by the methods of countercurrent distribution, paper chromatography, and bio-assay, the presence of estradiol-17 β in the ovaries of the dogfish *Squalus suckleyi* and the starfish *Pisaster ochraceus* (2, 3). Donahue (4) has shown the presence of estrogenic substances in the eggs of the lobster *Homarus americanus* and by chromatographic analysis has tentatively identified the presence of α -estradiol (5). The following report presents results obtained in an effort to isolate estrogens from the eggs of the American lobster using the methods commonly employed with mammalian material.

Materials and Methods

Lobster eggs (14,528 g) were obtained through the courtesy of the Fisheries Research Board of Canada. The eggs were stripped from the swimmerets and stored in approximately twice their volume of acetone until extraction. Then the acetone was poured off into large evaporating dishes; the eggs homogenized and allowed to stand overnight in acetone again. This acetone was then added to that already in the evaporating dishes and taken to dryness under a stream of warm air.

The oily residue remaining was dissolved in 95% ethyl alcohol. Distilled water was then added to dilute the alcohol to 70% and this was extracted three times with petroleum ether. The alcoholic solution was subsequently diluted to 35% and the above procedure was repeated using fresh petroleum

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Contribution from the Biological Laboratories, Harvard University, Cambridge, Massachusetts.

²Present address: The Biological Laboratories, Princeton University, Princeton, New Jersey.

ether. The 35% alcoholic fraction was reduced in volume by evaporation under a stream of warm air. A small amount of the solution remaining, when bio-assayed by the Astwood method (8), showed a slight but positive estrogenic reaction. The remainder of the crude 35% ethanol fraction was subjected to three different procedures.

(a) One-quarter was evaporated *in vacuo* and the residue was put in just enough 1 N KOH to dissolve it. Then it was extracted three times with ethyl ether. The alkali remaining was titrated to pH 7.5 with concentrated HCl and extracted three times with ether. The second ether extract was dried *in vacuo* and taken up in 15 ml methanol. One-milliliter aliquots were used for bio-assay purposes.

(b) One-quarter was evaporated *in vacuo* then hydrolyzed by refluxing for 1 hour in a solution 15% by volume HCl. Then the solution was neutralized with 2 N NaOH and evaporated to dryness. After this, procedure (a) outlined above was followed.

(c) One-half was partitioned between 70% methanol and a 50-50 mixture of chloroform and carbon tetrachloride in a 29-transfer countercurrent distribution. Five fractions were collected, which resulted from combining tubes 0-2, 3-9 (estrone), 10-19 (estradiol), 20-25 (estriol), 26-29, and were dried *in vacuo* (6, 7). The residue from each group of tubes was dissolved in 15 ml of methanol and 1-ml aliquots were taken from each for bio-assay of estrogenic potencies. Each aliquot was dissolved in 0.6 ml sesame oil and the alcohol evaporated. Rats of the Harvard strain, 21 days old, weighing 48-58 g were used as test animals. Two animals were used to test the material from each set of tubes. Each rat was given 0.2 ml of the sesame oil in a single subcutaneous dose and the uteri were removed and weighed 6 hours later. A similar bio-assay method was followed with the material obtained from procedures (a) and (b).

Results

(a) The 6-hour Astwood assay, using two animals, each receiving 0.2 ml sesame oil as a subcutaneous injection, proved negative. At a later time the remainder of the KOH-treated material when injected into a single rat resulted in a uterine wet weight of 30.7 mg (control 21.5 mg) showing a positive estrogenic response.

(b) The Astwood assay resulted in a positive response. Two rats given 0.2 ml sesame oil in a single subcutaneous injection had uteri weighing 31 and 30 mg at autopsy 6 hours later.

(c) Estrogenic activity was indicated only in the material obtained from tubes 10-19 (Table I). Fraction 10-19 was divided into five aliquots and subjected to a toluene-propylene glycol (1:1) chromatographic system at room temperature for 6 hours. The unknown fraction was applied to one strip, estrone to another, estradiol to a third, and a mixture of estrone and estradiol to a fourth. The four strips were run simultaneously. The three strips treated

with authentic estrogens were stained with ferric chloride - ferricyanide stain (7). Areas on the experimental strip corresponding to the standards were cut and eluted three times with 10-ml portions of acetone, ether, and absolute ethanol. The eluates were evaporated in part, transferred to small vials, and reduced to dryness. Sesame oil, 0.4 ml, was added to each vial and 0.1 ml was injected as a single subcutaneous dose into each of two 21-day-old rats. Results of the bio-assays (Table II) indicate the presence of estradiol-17 β . Test fractions corresponding to authentic estrone always gave negative results. Eluates of the paper outside of the test areas gave negative responses except for a slight response from the origin down to the estradiol-17 β position.

TABLE I
Results of bio-assay from countercurrent separation

Fraction	Uterine wet wt., mg	% increase over control
Controls*	21.5	
0-2	24.2	0
3-9	19.8	0
10-19	31.4	42.7
20-25	19.1	0
26-29	20.5	0

* Seventeen animals.

TABLE II
Bio-assay of eluted paper strips from toluene - propylene glycol chromatographic system

	Countercurrent fractions 10-19	
	Uterine wet wt., mg	% increase over control
Controls*	21.5	
Experimental strip corresponding to estrone	21.7	0
Experimental strip corresponding to estradiol	34.7	61

*Seventeen animals.

When all four strips were stained with ferric chloride - ferricyanide a blue area appeared on the experimental strip corresponding to estradiol-17 β on the two standard strips. No such spot could be detected in the region corresponding to estrone.

The bio-assay used here has been carefully standardized for the Harvard strain of rats. The minimal dose of estradiol which induces a 33% increase in uterine wet weight in 6 hours is 0.025 μ g, and of estrone, 0.45 μ g (8, 9). Using this comparison the lobster eggs on the swimmerets contain between 0.26 and 0.32 μ g of free estradiol-17 β per kilogram of fresh tissue.

Discussion

The results strongly indicate the presence of estradiol-17 β in the eggs of the American lobster, *Homarus americanus*. Estrone could not be detected. In

marked contrast to Donahue's (4) finding the noted concentration of estrogenic materials was much lower than values obtained for mammalian tissues, but 3 to 8 times higher than values obtained for the starfish (3). In all cases results are expressed as free estradiol per kilogram of wet tissue.

The presence of bound estrogenic material approximately equal to the free estradiol was indicated by methods (a) and (b) above. No further identification, by countercurrent studies or paper chromatography, was carried out for the bound material. Differences in total quantities of estrogenic materials noted in this study from those obtained by Donahue might be due to the different extraction procedures used or the developmental state reached by the eggs before extraction. The eggs extracted in this study had reached the stage of development where the eyes of the embryo were visible through the egg shell.

Acknowledgments

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THE INHIBITION BY ACETAZOLEAMIDE OF RENAL PHOSPHATE-ACTIVATED GLUTAMINASE IN RATS¹

JOHN. R. BEATON

With the technical assistance of T. ORME

Abstract

Intraperitoneal injection of acetazoleamide (10 mg/100 g body weight) in rats elicits a decreased activity of renal phosphate-activated glutaminase within 1 to 3 hours, followed by a return to normal activity at 8 hours, an increased activity at 16 hours, and a return to normal activity at 24 hours. Urinary excretion of ammonia in acetazoleamide-treated rats is decreased in the 8-hour period following injection (period of decreased enzyme activity) and increased in the subsequent 16-hour period (period of increased glutaminase activity). The net effect is increased urinary excretion of ammonia during the 24-hour period. Acetazoleamide inhibits renal phosphate-activated glutaminase in vitro, 100% inhibition being attained with an inhibitor:substrate ratio 3:1. Inhibition by acetazoleamide in vitro is eliminated with increasing L-glutamine concentration, 100% restoration of activity occurring with an inhibitor:substrate ratio of 1:3. Acetazoleamide had no significant effect upon hepatic phosphate-activated glutaminase and arginase activities in vivo nor upon urinary excretion of urea.

Introduction

Acetazoleamide is known to inhibit carbonic anhydrase (1), causing metabolic acidosis, and to possess diuretic properties (2). In addition, Owen *et al.* (3) have shown recently that, in patients with liver disease, acetazoleamide administration caused an increase in mean arterial ammonia concentration and a prompt twofold increase in the release of ammonia into the renal vein accompanied by a reduction in urinary excretion of ammonia and a rise in urinary pH.

Glutamine is the most abundant amino acid in plasma and most tissues and, among other roles, glutamine has been implicated as a precursor of urinary ammonia (4) by its breakdown to ammonia and glutamic acid. Two enzymes can effect this catabolism of glutamine:phosphate-activated glutaminase which occurs in numerous animal tissues (5), and pyruvate-activated glutaminase found in liver tissue (6). The latter enzyme is several times less active than the former (7) and its metabolic significance is not clear. The present studies were carried out to ascertain the effect of acetazoleamide on renal phosphate-activated glutaminase activity in vivo and in vitro and on hepatic phosphate-activated glutaminase in vivo. Effects of acetazoleamide on excretion of ammonia and urea and on hepatic arginase activity have also been investigated.

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Contribution from Defence Research Medical Laboratories, Toronto, Ontario.
Issued as DRML Report No. 109-5, PCC No. D50-93-10-72.

Methods

Adult male albino rats of the Wistar strain weighing 200–250 g and maintained on fox chow and drinking water ad libitum were used throughout these experiments. Experimental animals received acetazoleamide intraperitoneally in 0.5 ml distilled water containing an amount of acetazoleamide to give 10 mg/100 g body weight. Control animals were given 0.5 ml distilled water by intraperitoneal injection. At the periods of time indicated, the animals were killed by stunning and decapitation. For the measurement of renal phosphate-activated glutaminase activity, a kidney was rapidly removed, decapsulated, washed, and blotted. A portion of the kidney was weighed and homogenized in a Potter homogenizing tube employing a teflon pestle. The renal enzyme activity was measured by a modified Archibald method (8) and the necessary corrections for non-enzymatic hydrolysis of glutamine and for preformed ammonia were made as described by Beaton and Goodwin (9). Tissue homogenate was added to a solution containing phosphate buffer, glutamine, and, in the *in vitro* studies, acetazoleamide. The tube contents were incubated for 20 minutes at 38° C. There was no preincubation of tissue with either substrate or inhibitor prior to determination of enzyme activity. It was found that acetazoleamide *per se* reduces color formation in nesslerization. In the *in vitro* studies, corrections were made for this "damping" effect of acetazoleamide on color formation. Hepatic phosphate-activated glutaminase activity was determined in homogenates by the method of Archibald (10) as modified by Beaton and Ozawa (7). In one experiment, urinary urea was measured by the method of Archibald (11) and urinary ammonia nitrogen by a modification of the Conway method (12). Arginase activity was measured in liver homogenates by the procedure of Van Slyke and Archibald (13).

Significance of difference between means was tested by application of Student's "*t*" test.

Experimental

Effect of Acetazoleamide on Renal Glutaminase Activity In Vivo

One hundred and twenty rats were divided into five control and seven acetazoleamide-injected groups of 10 rats each. A control group was sacrificed at each of 0, 3, 8, 16, and 24 hours after injection; an acetazoleamide-injected group was sacrificed at each of ½, 1, 2, 3, 8, 16, and 24 hours after injection. Renal phosphate-activated glutaminase activities were determined and the results are shown in Fig. 1, each point with extensions representing the mean value and its standard error for 10 rats. Acetazoleamide administration caused a progressive fall in renal glutaminase activity reaching an apparent minimum value at about 2 to 3 hours after injection. This depression in activity was significant at 1 hour ($P < 0.02$), 2 hours ($P < 0.005$), and 3 hours ($P < 0.02$). The depressed activity was followed by a return to normal at 8 hours, a significantly increased activity at 16 hours ($P < 0.001$), and a return to normal at 24 hours.

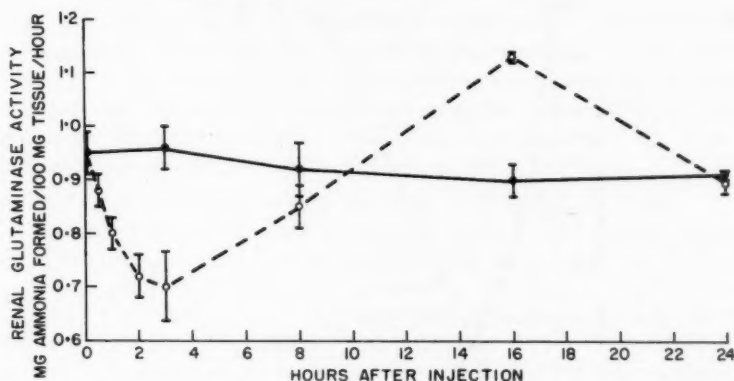


FIG. 1. Effect of acetazolesamide injection (10 mg/100 g body weight) on renal phosphate-activated glutaminase activity in vivo. Each point represents the mean glutaminase activity for 10 rats, the vertical bars depicting the standard error of the mean. Control groups (●—●); acetazolesamide-injected groups (○---○).

Effect of Acetazolesamide on Hepatic Glutaminase Activity In Vivo

One control and one acetazolesamide-injected group of 10 rats each were sacrificed 2 hours after injection, and hepatic phosphate-activated glutaminase activities were measured. The results, expressed in μg ammonia formed/100 mg tissue/hour as the mean value with its standard error were: control, 0.188 ± 0.012 ; acetazolesamide group, 0.164 ± 0.015 . The difference between these values is not statistically significant.

Effect of Acetazolesamide on Renal Glutaminase Activity In Vitro

In eight separate experiments, acetazolesamide was added to 1-ml aliquots of kidney homogenates at levels of 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 μmoles , each contained in 0.1 ml glass-distilled water. Reaction tubes contained 20 μmoles of the substrate L-glutamine, 20 mg of kidney tissue, and phosphate-cyanide buffer (pH 7.2). The enzyme activity was determined in the presence of these graded amounts of acetazolesamide and the results are shown in Fig. 2 as means and standard errors for eight determinations. It is apparent that, under these conditions, complete inhibition of the renal enzyme is obtained with addition of 60 μmoles acetazolesamide, i.e. at an inhibitor:substrate ratio of 3:1. Addition of acetazolesamide did not alter the pH of the buffered solutions; the effect of acetazolesamide is therefore not due to an alteration in pH. In six experiments, graded amounts of L-glutamine (5–100 μmoles) were added to reaction tubes containing 20 μmoles acetazolesamide, 20 mg kidney tissue, and phosphate-cyanide buffer (pH 7.2). The results of renal glutaminase activity determinations are shown in Fig. 2 as means and standard errors for six determinations. It is apparent that the inhibition of activity by 20 μmoles acetazolesamide is completely eliminated by addition of 60 μmoles L-glutamine, i.e. at an inhibitor:substrate ratio of 1:3. It is noted

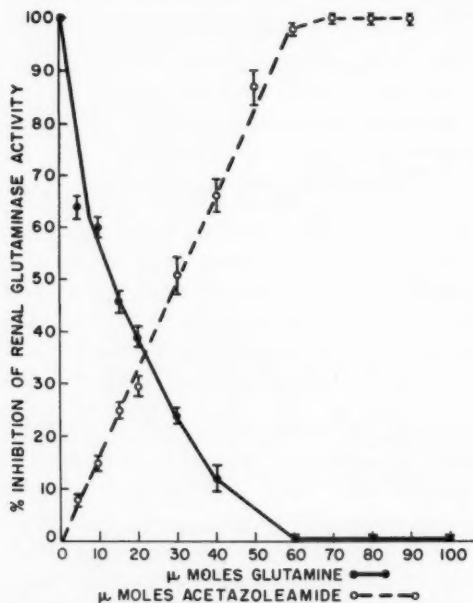


FIG. 2. Effect of L-glutamine level (●—●) on renal glutaminase inhibition by 20 μ moles acetazolesamide and effect of acetazolesamide level (○---○) on renal glutaminase activity in the presence of 20 μ moles L-glutamine. Each reaction tube contained 20 mg kidney tissue in potassium cyanide-phosphate buffer (pH 7.2) and either 20 μ moles L-glutamine or acetazolesamide; incubated 20 minutes at 37° C.

from the data of Fig. 2 that the two curves intersect at an inhibitor:substrate ratio of approximately 1:1. We have observed that 100% inhibition of activity occurs with an inhibitor:substrate ratio of 3:1 with the L-glutamine level at 10 and 30 μ moles in addition to the 20 μ moles described already. Conversely, inhibition is completely eliminated by a substrate:inhibitor ratio of 3:1 with the acetazolesamide level at 10 and 30 μ moles in addition to the 20 μ moles described already. Attempts to determine the type of inhibition by classical Lineweaver-Burk plots (14) have not been fruitful, suggestive that more than a single enzyme may be affected under our experimental conditions. This could be a consequence of using a kidney homogenate in contrast to using an isolated, purified enzyme. It should be noted that ammonia formation from amino acids and asparagine is largely eliminated by the potassium cyanide in the buffer although ammonia production from codehydrogenase I, adenosine, and guanosine is not eliminated (10).

Effect of Acetazolesamide on Hepatic Arginase Activity In Vivo

At each of 1 and 2 hours after injection, one control and one acetazolesamide-injected group of 10 rats each were sacrificed and hepatic arginase activities were determined. The results expressed in μ moles urea formed/100 mg tissue/

hour as the mean with its standard error were: control (1 hour) 59 ± 6.5 ; acetazoleamide (1 hour) 65 ± 2.6 ; control (2 hours) 67 ± 5.1 ; acetazoleamide (2 hours) 69 ± 3.6 . There is no significant difference between mean values of control and acetazoleamide-treated groups at either the 1- or 2-hour period. Thus, under these experimental conditions, acetazoleamide did not alter hepatic arginase activity *in vivo*.

Effect of Acetazoleamide on Urinary Excretion of Urea and Ammonia

Two groups of 10 animals each were placed in small, individual metabolism cages. One group was given 0.5 ml distilled water by intraperitoneal injection. The second group was given acetazoleamide (10 mg/100 g body weight). Throughout the next 24 hours, fox chow and drinking water were provided ad libitum and urine was collected under toluene. At the end of this period, urine volume and water consumption were measured and the animals were sacrificed for determination of renal glutaminase activities. It is recognized that precise measurement of water consumption and urine volume in metabolism cages is difficult and potential errors exist. However, both groups were treated in exactly the same manner, with the exception of acetazoleamide injection, so that small errors would be expected to be about equal in the two groups and would not, therefore, alter the findings with respect to the acetazoleamide injection. Urea and ammonia concentrations in the urine samples were determined. In addition, kidneys were weighed and the values expressed as percentage of body weight. The results of this study are shown in Table I.

TABLE I
Effect of acetazoleamide on renal glutaminase activity and urinary excretion of urea and ammonia (results expressed as mean \pm standard error of the mean for 10 rats)

Group	Control	Acetazoleamide-injected	Probability, <i>P</i>
Kidney weight, % body weight	0.78 ± 0.021	0.79 ± 0.026	—
Water intake, ml/24 hours	24 ± 2.5	30 ± 3.3	—
Urine volume, ml/24 hours	8 ± 0.6	18 ± 2.7	<0.01
Urinary urea,			
mg/ml	134 ± 20	57 ± 9	<0.001
mg/24 hours	928 ± 81	800 ± 65	—
Urinary ammonia N,			
mg/ml	0.87 ± 0.11	0.69 ± 0.10	—
mg/24 hours	6.02 ± 0.44	9.92 ± 1.10	<0.01
Renal glutaminase activity*	0.91 ± 0.003	0.90 ± 0.019	—

*Mg ammonia formed/100 mg tissue/hour. Enzyme activity determined 24 hours after injection of water or acetazoleamide.

Under these conditions, and indicative of its diuretic property, acetazoleamide caused a significant increase in 24-hour urine volume but not in water consumption. Acetazoleamide effected a significant decrease in urinary urea concentration but not in total output of urinary urea and a significant increase in total urinary output of ammonia but not in urinary ammonia concentration.

Twenty-four hours after administration of acetazoleamide, the mean renal phosphate-activated glutaminase activity was not different from that of control animals. Acetazoleamide did not alter kidney weight in relation to body weight.

Effect of Acetazoleamide on Urinary Excretion of Ammonia During a 24-Hour Period

Nine control and nine acetazoleamide-injected rats were placed in metabolism cages, and food and water were provided ad libitum. Due to the relatively small volume of urine excreted by control rats in an 8-hour period, it was necessary to place three animals in each cage in order to collect sufficient samples for analysis and to minimize losses and consequent errors. Urines were collected under toluene for the periods 0-8 and 8-24 hours after injection and were pooled by group for each period. Volumes were recorded and ammonia determinations were carried out. The results expressed as the average for each group of nine rats (pooled samples) are shown in Table II. It is

TABLE II
Effect of acetazoleamide on urinary excretion of ammonia during a 24-hour period
(results expressed as mean value for nine rats)

Group	Period after injection, hours	Urine volume, ml/rat/hour	Urine ammonia N	
			mg/ml	mg/rat/hour
Control	0-8	0.20	0.79	0.19
Acetazoleamide-injected	0-8	0.83	0.043	0.037
Control	8-24	0.21	1.25	0.26
Acetazoleamide-injected	8-24	0.53	1.49	0.79
Control	0-24	0.21	1.09	0.23
Acetazoleamide-injected	0-24	0.63	1.01	0.64

evident that urine volume was markedly increased in acetazoleamide-treated rats in both time periods. However, ammonia concentration and total ammonia excretion were less in the 0- to 8-hour period and more in the 8- to 24-hour period than were the values for control groups. The net effect over the 24-hour period was a decreased urinary ammonia concentration and increased ammonia excretion in acetazoleamide-treated rats, as observed in the preceding experiment.

Discussion

It is evident that acetazoleamide inhibits renal phosphate-activated glutaminase in vitro. This inhibition can be eliminated by an increased concentration of the substrate L-glutamine. In vivo, acetazoleamide depresses renal glutaminase activity initially but this depression is followed by an increased activity or "rebound effect".

It is of considerable interest that urinary excretion of ammonia in acetazoleamide-treated rats follows the same pattern in time as does renal glu-

taminase activity, i.e. decreased ammonia excretion in the period of decreased enzyme activity and vice versa. Glutamine has been implicated as a major precursor of urinary ammonia (4). The present results support this and suggest that glutamine performs this function as a consequence of the action of renal phosphate-activated glutaminase. The data in Table I show that kidney weight is not altered by acetazoleamide treatment so that the increased urinary excretion of ammonia is not a consequence of increased kidney mass. Further, the amino group of acetazoleamide, even if converted to ammonia, which is doubtful, could not account per se for more than 14% of the increased ammonia excretion of the rats treated with the dosage used in these studies. The observation of an initial decline in urinary excretion of ammonia is in accord with the finding of Owen *et al.* (3) in human patients, but, over a longer period, the net effect of acetazoleamide treatment is an increase in urinary excretion of ammonia.

The apparent lack of effect of acetazoleamide on hepatic phosphate-activated glutaminase and arginase activities is of interest and, with respect to arginase, is consistent with the unaltered urinary excretion of urea of treated rats.

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A STUDY ON THE LIBERATION OF SUGARS, HEXURONIC ACID, AND HEXOSAMINE FROM BIOLOGICAL MATERIAL¹

W. HAAB² AND P. A. ANASTASSIADIS

Abstract

Sulphonated polystyrene resin suspended in dilute hydrochloric acid and 4 *N* hydrochloric acid were compared with respect to (a) efficiency as catalysts of hydrolytic cleavage of mucopolysaccharides and (b) rates of destruction of sugars, glucuronic acid, and glucosamine. Mannose, galactose, and glucose were not subject to serious destruction when heated with the resin suspension over a period that was sufficient to effect hydrolysis of mucopolysaccharides and glycoproteins. All these sugars were subject to serious destruction when heated with 4 *N* hydrochloric acid for a period sufficient to effect hydrolysis of mucopolysaccharides and glycoproteins. Under similar conditions, pentoses were destroyed more rapidly than hexoses but ribose withstood heating with the resin suspension for several hours. Heparin was found to be more resistant to hydrolysis than chondroitin sulphate or hyaluronic acid.

Introduction

It was shown in previous work (1) that a suspension of a polystyrene resin in weak hydrochloric acid presents some advantages over strong hydrochloric acid as a catalytic agent for the hydrolysis of mucopolysaccharides and tissues. The principal advantage of the resin-catalyzed hydrolysis was relatively slight destruction of hexuronic acid. It was shown, furthermore, that the conditions of hydrolysis (temperature, concentration of acid in which the resin was suspended, time of heating) influenced substantially the rates of liberation of hexosamine and hexuronic acid and the degree of destruction of hexuronic acid. It was considered advisable, therefore, to investigate further the potentialities of resin hydrolysis with the following objectives:

(a) To study the possibility that monosaccharides participating in the glycoprotein complex are liberated more effectively with resin hydrolysis than with strong acid hydrolysis. Since the principal shortcoming of any acid hydrolysis of glycoproteins is the rapid degradation of the sugars and sugar acids when heated with acids, it was decided to investigate the rates of destruction of the most common monosaccharides when they were heated with strong hydrochloric acid and with resin - weak acid suspension.

(b) To study the effects of temperature and the concentration of hydrochloric acid in the resin suspension on the rate of hydrolysis of mucopolysaccharides and on the rate of destruction of the hydrolysis products. Since hexosamine is a universal constituent of mucopolysaccharides and has been found to be fairly resistant to acid hydrolysis, the degree of liberation of

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²Present address: Department of Chemistry, University of Scranton, Scranton, Pennsylvania, U.S.A.

hexosamine was considered to be a reliable indication of the rate of hydrolysis of a mucopolysaccharide. On the other hand, since hexuronic acid is more labile to acid than either hexosamine or hexoses, the degree of destruction of glucuronic acid was considered to be an indication of the rate of destruction of the labile product of hydrolysis.

(c) To study the rates of hydrolysis of different kinds of mucopolysaccharides and especially of chondroitin sulphate, heparin, and hyaluronic acid with 4 *N* hydrochloric acid and with resin - weak hydrochloric acid suspension.

Experimental

(A) Analytical Methods

All resin-catalyzed hydrolyses were performed in sealed pyrex tubes which were rotated end-over-end in an air oven fitted with a circular plate that rotated in a vertical plane. This oven was a modification of a specific asphalt-testing oven manufactured by the Blue M Electric Company, Blue Island, Illinois. Hexosamine was determined by the *p*-dimethylaminobenzaldehyde reaction (2), hexuronic acid by the naphthoresorcinol reaction (3), hexoses by the anthrone reaction (4), ribose by the orcinol reaction (5), and the deoxy-ribose by the indole reaction (6).

(B) The Course of Destruction of Some Monosaccharides when Heated with Resin/0.05 *N* HCl Suspension or with 4 *N* HCl

Preliminary Work on the Hexoses

The amount of hexoses remaining presumably unaltered after being heated was determined by the anthrone reaction (4). In order to investigate whether hexoses are preferably determined directly in the heated mixture, or after neutralization of the mixture, or after elimination of the hydrochloric acid from it, the effect of the presence of chloride and sodium ions on the intensity of the color produced by the anthrone reaction was studied. The relative absorbances obtained (absorbance of glucose dissolved in water taken as 100) when glucose was dissolved in solutions of hydrogen chloride, and in solutions of sodium chloride, are given in Table I. The results show that the color produced by the glucose/anthrone reaction is extremely sensitive to chloride ions but that it is not affected appreciably by the presence of sodium ions.

TABLE I
Effect of chloride and sodium ions on the intensity of the color produced by glucose in the anthrone reaction

Composition of solvent	Relative absorbance	Composition of solvent	Relative absorbance
Water	100.0	Water	100.0
0.01 <i>N</i> HCl	108.7	—	—
0.2 <i>N</i> HCl	139.5	0.2 <i>N</i> NaCl	138.1
0.4 <i>N</i> HCl	154.1	0.4 <i>N</i> NaCl	153.8
0.8 <i>N</i> HCl	179.1	0.8 <i>N</i> NaCl	181.3
1.8 <i>N</i> HCl	189.5	1.8 <i>N</i> NaCl	211.3

Over the range tested, an increase in the concentration of chloride ions produced non-proportional increase in the intensity of the color. Elimination of hydrochloric acid after any treatment of the sample with hydrochloric acid (e.g. hydrolysis) is, therefore, necessary for a reliable determination of hexoses by the anthrone reaction. A convenient way to perform this elimination is by placing the sample in a vacuum desiccator over solid sodium hydroxide under reduced pressure.

The effect of time of heating on the intensity of color produced by the mannose and galactose with the anthrone reagent was studied and the results are presented in Table II. The optimal time of heating of glucose with anthrone

TABLE II
Effect of time of heating on the intensity of the color produced
by mannose and galactose in the anthrone reaction

Heating time (minutes)	Relative absorbance		Heating time (minutes)	Relative absorbance	
	Mannose	Galactose		Mannose	Galactose
2	56.7	56.2	10	—	100.0
3	84.6	—	12	79.2	93.1
4	100.0	95.3	14	—	81.9
5	100.0	—	16	63.5	74.3
6	96.9	97.5	20	—	61.3
7	88.6	—	25	2.9	—
8	89.2	100.0	—	—	—

reagent was given in reference 4 as 12 minutes. As is shown in Table II, the optimal time of heating of mannose and galactose with the anthrone reagent was found to be 4–5 minutes for mannose and 8–10 minutes for galactose. However, no difference between the shape of the absorption curves of the color given by mannose or galactose with anthrone and the shape of the curve given by glucose with anthrone was detected. The ratio concentration:absorbance for mannose and galactose decreased for concentrations above 160 $\mu\text{g}/\text{vessel}$ and therefore the best range of concentration would appear to lie between 75 and 150 $\mu\text{g}/\text{vessel}$.

The Course of Destruction of Aldohexoses

Glucose (0.5 mg and 2 mg), mannose (1 mg), and galactose (1 mg) were heated at 100° C, for different time intervals with 4 *N* HCl and resin/0.05 *N* HCl in the oven described above, with the vertical plate rotating at 4–6 revolutions per minute. The destructions of sugars (expressed as a percentage of the amount originally present) for different times of heating are given in Table III. Of the three principal natural aldohexoses, mannose was destroyed fastest when heated with 4 *N* HCl followed by galactose, and the most resistant one was glucose. This is easily understood because the configuration of glucose is more symmetrical than the configuration of the other two sugars and because the hydroxyl groups of galactose are better balanced than the hydroxyl groups of mannose. The data of Table III indicate further that the degradations of

TABLE III

Percentage destruction of glucose, mannose, and galactose when heated at 100° C with 4 *N* HCl (acid) and resin/0.05 *N* HCl (resin) for different time intervals

Heating time	Glucose		Mannose		Galactose	
	Acid	Resin	Acid	Resin	Acid	Resin
0 min	0.0	0.0	0.0	0.0	—	0.0
30 min	2.0	—	0.0	0.0	2.6	0.0
1 hr	5.7	2.0	6.8	—	3.1	0.0
2 hr	17.5	2.0	26.7	0.0	14.0	0.0
4 hr	36.1	1.0	60.2	0.0	46.6	0.0
8 hr	60.7	0.1	82.7	0.0	67.5	0.0
16 hr	—	6.1	100.0	0.0	97.4	0.0
24 hr	—	6.1	—	0.0	100.0	0.0
32 hr	—	10.9	—	—	—	—
40 hr	—	—	—	3.5	—	5.5
48 hr	—	—	—	6.0	—	7.5
72 hr	—	—	—	14.4	—	14.4
96 hr	—	—	—	22.3	—	21.4

mannose, galactose, and glucose were substantially slower when the hexoses were heated with resin/0.05 *N* HCl suspension than when they are heated with 4 *N* HCl. A given degree of hydrolysis of a biological material calls, at the most, for 10 times as long a period of heating (usually it calls for much shorter heating) with resin/0.05 *N* HCl as with 4 *N* HCl. It may be concluded, therefore, that mannose, galactose, and glucose may be liberated from mucopolysaccharides or glycoproteins containing them more effectively with resin hydrolysis than with 4 *N* HCl hydrolysis.

The Course of Destruction of Pentoses

The courses of destruction of D-ribose and D-deoxyribose when heated with 4 *N* HCl and with resin/0.05 *N* HCl at 100° C and at 90° C were followed by the determination of D-ribose by the orcinol reaction (5) and by the determination of the deoxyribose by the indole reaction (6). The relevant data are presented in Table IV. It appears that pentoses were destroyed much more easily than hexoses but that deoxyribose was resistant to destruction when heated with resin/0.05 *N* HCl at 100° C for at least 8 hours.

The Course of Destruction of Glucosamine and Glucuronic Acid

The percentage destruction of the above substances when heated at different intervals, as determined by the *p*-dimethylaminobenzaldehyde reaction (2) and naphthoresorcinol reaction (3), are given in Table V. It will be noted that glucosamine was resistant to both strong hydrochloric acid and resin hydrolysis. However, for prolonged and comparable periods of hydrolysis, glucosamine seemed to have been somewhat more readily destroyed in the course of resin hydrolysis than in the course of hydrolysis with 4 *N* HCl.

The rate of destruction of glucuronic acid when heated with resin/0.05 *N* HCl at 100° C was found to be the same as that reported previously (1), but at 90° C the rate of destruction of glucuronic acid when heated with resin was

TABLE IV
Percentage destruction of D-deoxyribose and D-ribose when heated with 4 N HCl (acid) and resin/0.05 N HCl (resin)

Heating time	D-Deoxyribose			D-Ribose	
	Acid, 100° C	Resin		Acid, 100° C	Resin, 100° C
		90° C	100° C		
0 min	83.2	—	12.7	0.0	0.0
15 min	—	—	—	20.7	—
30 min	100.0	50.4	59.4	40.6	0.0
1 hr	—	—	—	64.6	0.0
2 hr	—	94.7	94.8	90.3	0.0
3 hr	—	—	—	96.3	—
4 hr	—	96.4	95.4	—	0.0
8 hr	—	97.1	—	—	2.8
16 hr	—	—	—	—	35.0
24 hr	—	—	—	—	35.7

substantially lower than at 100° C. In the cases of mucopolysaccharides or glycoproteins that liberate their glucuronic acid relatively easily, heating with resin suspension at 90° C, therefore, may be preferable to heating at 100° C. The rates of destruction of glucuronic acid when heated with resin/0.25 N HCl and when heated with resin/0.05 N HCl were about the same. This result differs from the results obtained in the hydrolysis of heparin, where lower values of hexuronic acid were obtained with resin/0.25 N HCl than with resin/0.05 N HCl. Since resin/0.25 N HCl suspension is more effective as a catalyst than resin/0.05 N HCl, this point is worthy of further investigation.

TABLE V
Percentage destruction of glucosamine and glucuronic acid when heated with 4 N HCl (acid), resin/0.05 N HCl (resin/0.05), and resin/0.25 N HCl (resin/0.25)

Heating time	Glucosamine			Glucuronic acid			
	Acid, 100° C	Resin/0.05		Acid, 100° C	Resin/0.05		Resin/0.25, 100° C
		90° C	100° C		90° C	100° C	
0 min	0.0	—	0.0	22.0	0.0	0.0	0.0
30 min	0.0	—	0.0	24.0	—	5.4	—
2 hr	0.0	—	0.0	54.8	0.0	7.0	1.1
3 hr	0.0	—	—	81.0	—	—	—
4 hr	0.0	—	0.0	83.5	0.0	9.0	1.1
6 hr	0.0	—	—	87.9	—	—	—
8 hr	0.0	—	0.0	93.5	0.3	9.0	6.1
12 hr	—	—	0.0	—	—	9.4	—
16 hr	0.0	0.0	0.0	99.7	0.3	15.4	12.9
24 hr	0.0	0.0	1.1	99.9	1.6	23.7	22.5
32 hr	—	—	3.0	—	3.2	—	28.9
36 hr	—	—	—	—	—	28.0	—
40 hr	—	—	—	—	6.1	—	37.3
48 hr	—	0.0	15.0	—	13.3	39.9	37.3
72 hr	32.7	—	17.0	100.0	17.3	48.0	39.7
96 hr	33.3	0.0	22.5	—	20.4	58.9	60.1
120 hr	—	1.9	36.0	—	24.1	62.2	67.4
168 hr	—	3.3	74.0	—	36.1	79.2	83.3

(C) The Course of Hydrolysis of Some Mucopolysaccharides

Heparin (Nutritional Biochemicals) was selected for a study on the catalytic efficiency and on the destructive capacity of the 4 *N* HCl and of resin - dilute hydrochloric acid suspensions. Portions, 0.5 mg and 5 mg each, of heparin and 5 ml 4 *N* HCl were heated in sealed pyrex tubes (13×100 mm) at 100° C for intervals of time indicated in Table VI. After the hydrolyzates were dried in

TABLE VI

Hexosamine (base, mg/g) and hexuronic acid (mg/g) recorded in strong acid hydrolyzates of samples of heparin (content of hydrolysis tube 0.5 mg heparin and 5 ml 4 *N* HCl, A, and 5 mg heparin and 5 ml 4 *N* HCl, B)

Time of heating at 100° C	Hexosamine values				Hexosamine relative values after chromatography		Hexuronic acid values after chromatography, B
	Before chromatography		After chromatography				
	A	B	A	B	A	B	
0 min	5.3	4.3	6.6	0.0	4.3	2.7	67.5
5 min	11.9	—	0.0	—	0.0	—	—
10 min	26.2	—	6.6	—	4.3	—	—
30 min	58.1	58.4	22.4	62.5	14.7	39.2	31.3
60 min	95.0	—	53.1	—	34.8	—	—
90 min	103.5	—	58.0	—	38.0	—	—
2 hr	121.2	161.8	86.3	139.4	56.5	87.3	18.8
3 hr	157.0	156.0	137.8	148.4	90.2	93.0	10.8
4 hr	167.7	179.1	152.7	159.7	100.0	100.0	8.0
6 hr	160.7	174.4	137.0	143.0	89.7	89.6	4.0
8 hr	164.3	—	117.9	160.6	77.2	100.6	3.0
16 hr	150.1	—	151.1	170.0	99.0	106.5	0.0
24 hr	141.1	—	118.7	150.2	77.7	94.1	—
48 hr	105.2	—	111.2	—	72.8	—	—
72 hr	139.1	—	132.0	130.0	87.0	81.4	0.0
92 hr	—	—	—	128.6	—	80.6	0.0

desiccators over sodium hydroxide under reduced pressure and the residues were redissolved in water, aliquots were analyzed for hexosamine directly or after chromatography on polystyrene resin columns (7). The results of these analyses are given in Table VI. The hexosamine values indicated that at least 4 hours are necessary for the hydrolysis of heparin with 4 *N* HCl. A comparison between the values before and after chromatography showed that a substantial part of the sources of irrelevant absorption was removed through chromatography. The hexuronic acid values, obtained in the water eluates of the hydrolyzates of the 5 mg samples, verified that the conditions of the 4 *N* HCl hydrolysis led to rapid destruction of hexuronic acid.

Portions of heparin (5 mg each) were heated in the rotary oven with 1+2 resin/water (R/W), or resin/0.05 *N* HCl (R/0.05), or resin/0.25 *N* HCl (R/0.25) suspensions for the intervals of time and the temperatures indicated in Table VII. The resin was Dowex-50 with a cross linkage 12, 200-400 mesh. After a double chromatography (1), which was done in chromatographic tubes 10×300 mm with sealed-in coarse porosity fritted disks (pyrex 38450),

hexosamine and hexuronic acid were determined in the hydrochloric acid and water eluates. The values obtained for hexosamine in hydrochloric acid eluates and for hexuronic acid in the water eluates are given in Table VII.

TABLE VII

Hexosamine (base, mg/g) and hexuronic acid (mg/g) recorded in resin hydrolyzates of samples of heparin (content of hydrolysis tube 5 mg heparin and 5 ml resin suspension)

Time of heating	Hexosamine				Hexuronic acid			
	R/W, 100° C	R/0.05, 100° C	R/0.25, 100° C	R/0.05, 90° C	R/W, 100° C	R/0.05, 100° C	R/0.25, 100° C	R/0.05, 90° C
0 min	0.5	0.0	0.0	—	37.2	88.6	69.8	99.4
30 min	0.4	0.0	0.0	—	29.7	53.6	60.0	—
2 hr	0.2	0.0	1.9	—	33.6	76.9	63.9	97.9
4 hr	0.0	0.8	11.1	—	27.3	74.8	99.0	100.8
8 hr	1.1	11.2	36.3	—	48.3	136.0	106.2	90.1
12 hr	—	52.0	106.9	—	—	—	110.6	—
16 hr	13.2	82.6	124.6	—	36.5	134.4	106.5	94.0
24 hr	16.0	128.7	191.2	—	38.9	110.7	95.9	95.5
32 hr	—	—	—	—	—	—	—	91.5
36 hr	26.5	139.8	217.7	—	76.7	42.3	103.1	—
40 hr	—	—	—	18.4	—	—	—	81.3
48 hr	47.6	—	196.2	25.3	70.1	—	67.8	74.3
72 hr	72.2	—	182.1	68.2	43.1	—	51.7	70.0
96 hr	74.4	150.3	176.1	106.3	22.6	36.7	23.3	60.2
120 hr	—	—	182.6	134.7	22.1	—	24.4	46.5
168 hr	—	—	148.3	113.6	15.0	—	10.0	42.6

The resin/0.25 *N* HCl hydrolyzed the heparin more completely than did either the resin/0.05 *N* HCl suspension or the 4 *N* HCl and the rate of hydrolysis was much faster with resin/0.25 *N* HCl suspension than with resin/0.05 *N* HCl suspension. It is interesting to note that the maximum hexosamine value obtained with resin/0.25 *N* HCl hydrolysis (217.7 mg/g at 36-hour heating) was substantially higher than the maximum value obtained with 4 *N* HCl hydrolysis after the elimination of interfering substances through chromatography (170 mg/g at 14-hour heating). The higher maximum for hexuronic acid was obtained when heparin was heated with resin/0.05 *N* HCl (for 16 hours), whereas substantially lower values were obtained with resin/0.25 *N* HCl. As it was mentioned above, this last result is at variance with the data presented in Table V, which indicated that there was not any fundamental difference in the rate of destruction of glucuronic acid whether it was heated with resin/0.05 *N* HCl or with resin/0.25 *N* HCl.

Portions of chondroitin sulphate (10 mg each) and of hyaluronic acid (0.733 mg each) (both Nutritional Biochemicals) were hydrolyzed for different time intervals with 5 ml 4 *N* HCl. The hexosamine values, obtained in the hydrochloric acid eluates of the chromatographed hydrolyzates, are given in Table VIII. In the first period of heating, both chondroitin sulphate and hyaluronic acid were hydrolyzed faster than heparin. It is probable that this difference in the original rate of hydrolysis was connected with the different rates of the splitting of the acetyl group from the amino group of the hexos-

TABLE VIII

Hexosamine (base, mg/g, and (or) relative values) recorded in strong acid hydrolyzates of samples of chondroitin sulphate (10 mg), of hyaluronic acid (0.733 mg), and of heparin (0.5 mg, A, and 5 mg, B)

Time of heating	Chondroitin sulphate		Hyaluronic acid		Heparin (relative values)	
	mg/g	Relative values	mg/g	Relative values	A	B
0	5.1	3	6.2	7	4	3
5 min	74.6	47	—	—	0	—
10 min	101.7	65	—	—	4	—
15 min	—	—	48.8	58	—	—
30 min	118.5	75	54.0	64	15	39
60 min	142.1	90	69.4	82	35	—
90 min	143.2	91	—	—	38	—
2 hr	139.1	88	70.4	83	57	87
3 hr	157.5	100	81.5	97	90	93
4 hr	157.5	100	84.5	100	100	100
6 hr	137.7	87	84.5	100	90	90
8 hr	—	—	86.4	103	77	101
12 hr	163.3	104	—	—	—	—
16 hr	—	—	85.5	101	99	107
24 hr	162.7	103	64.5	76	78	94
48 hr	—	—	46.8	55	73	—
72 hr	—	—	40.6	48	87	82
96 hr	—	—	—	—	—	81

amines of chondroitin sulphate and hyaluronic acid and of the sulphate group from the amino group of the hexosamine of heparin. The faster this splitting occurs, the slower the original rate of hydrolysis. The above results, therefore, may be considered as an indication that the N-sulphate linkage is split faster than the N-acetyl linkage and that the electrostatic shield (8) is established earlier in the case of heparin. As the time progressed, however, the difference in degree of hydrolysis between chondroitin sulphate and heparin was not so great. This was to be expected, because once the shield had been established the rates of hydrolysis of the two mucopolysaccharides were unlikely to be very different. However, in the interval between 3 and 4 hours from the beginning of heating, no additional hexosamine was liberated from the chondroitin sulphate, whereas additional hexosamine was liberated from heparin.

It is difficult to decide from the data whether chondroitin sulphate or hyaluronic acid was hydrolyzed the faster because, as the data for heparin show, the ratio mucopolysaccharide/hydrochloric acid influenced the original rate of hydrolysis and in these experiments the ratio for chondroitin sulphate was 15 times larger than the ratio for hyaluronic acid.

(D) Some Comparisons between Strong Hydrochloric Acid Hydrolyzates and Resin - Weak HCl Hydrolyzates

Table IX gives values for hexoses and hexuronic acid obtained when certain biological materials were hydrolyzed with 4 N HCl for 4 hours and with resin/0.25 N HCl for 36 hours. The values given for the 4 N HCl hydrolyzates were

TABLE IX

Hexoses and hexuronic acid (mg/g) found in some materials after 4 N HCl hydrolysis and resin/0.25 N HCl hydrolysis

Material	Hexoses		Hexuronic acid	
	4 N HCl hydrolysis	Resin hydrolysis	4 N HCl hydrolysis	Resin hydrolysis
Avian magnum	10.6	25.2	2.0	4.6
Avian skin	15.4	27.8	2.8	4.2
Bovin α -casein	2.4	4.9	1.5	1.5
Avian egg-white	12.6	75.0	3.4	7.2
Avian egg-yolk	12.6	24.2	1.3	2.0
Heparin	—	—	13.7	52.5
Chondroitin sulphate	—	—	35.0	88.8

obtained after chromatography through resin in the water eluates. The values obtained before chromatography were usually higher, indicating that substances interfering with the analyses had been eliminated through chromatography. The data of Table IX indicate that, for materials tested, the resin hydrolysis liberated more effectively the hexoses and hexuronic acid. However, in order to evaluate the effect of chromatography, and especially to compare the two ways of hydrolysis, the absorption curves of the colors obtained for the determination of sugars and hexuronic acid were studied.

An inspection of the absorption curves given by the anthrone reaction (see Fig. 1) leads to the following conclusions. In the cases studied the shape of the curves obtained when the hydrochloric acid hydrolyzates (after removal of the hydrochloric acid) are heated with the anthrone reagent deviated substantially from the shape of the curves obtained when hexose standards were treated with the anthrone reagent (compare curve 2 with curve 1 of Fig. 1). Most of these deviations were removed by resin chromatography (compare curves 3 and 2 with curve 1). The shape of the curves obtained when the water eluates of the resin hydrolyzates were treated with anthrone reagent was practically identical with the shape of the curves for the standards (compare curve 4 with curve 1). The absorbances given at 620 $m\mu$ (region of maximal absorbance of the standard) were 2 to 6 times higher when the water eluates of the resin hydrolysis were treated with anthrone reagent than when the 4 N HCl hydrolyzates, either before or after chromatography, were treated with anthrone reagent (compare curve 4 with curves 2 or 3).

An inspection of the absorption curves given by the naphthoresorcinol reaction leads to the following conclusions. Very small fractions of the absorbances at 600 $m\mu$ obtained when the unchromatographed 4 N HCl hydrolyzates of the materials were treated with naphthoresorcinol may be attributed to the hexuronic acid (compare, for instance, curve 1 with curve 3 in Fig. 2). After chromatography a part of the irrelevant absorption was removed but even then only a small portion of the absorbance at 600 $m\mu$ could be attributed to hexuronic acid (compare curve 2 with curve 3). On the contrary, a very large part of the absorbance given after the resin hydrolysis of the materials

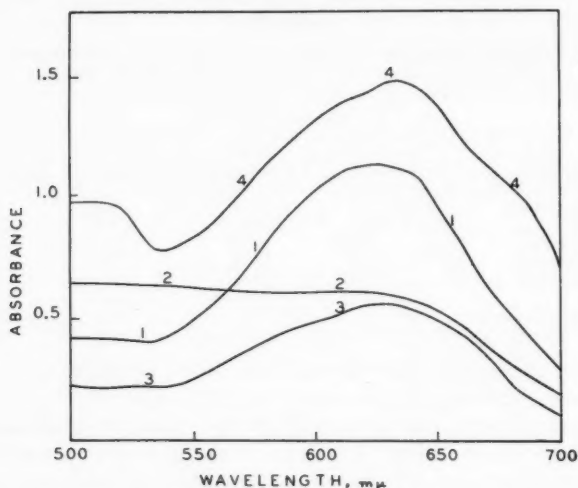


FIG. 1. Anthrone reaction absorption curves given by 20 mg avian oviduct magnum per reaction vessel (content of vessel 12 ml) and by a mixture of hexoses (glucose + mannose + galactose). Absorption cell 1 cm.

Curve 1, hexoses 200 μ g per vessel. Curve 2, magnum after 4 *N* HCl hydrolysis without chromatography. Curve 3, magnum after 4 *N* HCl hydrolysis and chromatography. Curve 4, magnum after resin hydrolysis.

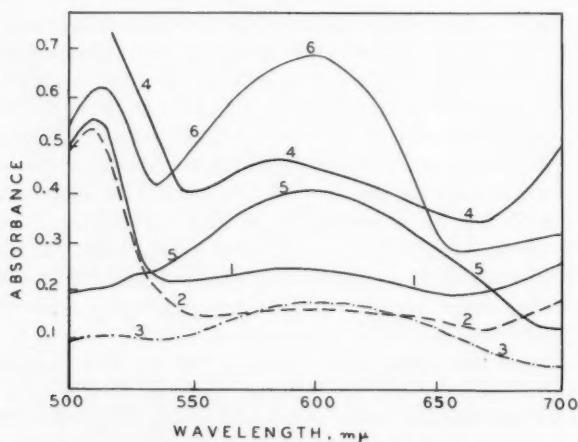


FIG. 2. Naphthoresorcinol reaction absorption curves given by 10 mg of avian oviduct magnum per reaction vessel (final volume of colored solution 11 ml) and by glucuronic acid. Absorption cell 1 cm.

Curve 1, magnum after 4 *N* HCl hydrolysis without chromatography. Curve 2, magnum after 4 *N* HCl hydrolysis and chromatography. Curve 3, glucuronic acid 20 μ g per vessel. Curve 4, magnum after resin hydrolysis. Curve 5, glucuronic acid 40 μ g per vessel. Curve 6, glucuronic acid 80 μ g per vessel.

was undoubtedly derived from the hexuronic acid content of the materials. However, part of the absorbance may also be attributed to interferences (compare curve 4 with curves 5 and 6). It is difficult to determine only from the curves that proportion of the total absorbance at 600 m μ which was irrelevant absorption. Further work, therefore, will be necessary in order to devise a procedure to eliminate these interferences. In analyses of pure mucopolysaccharides the amount of such interferences is very small.

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EFFECTS OF DIETARY METHIONINE AND VITAMIN B₁₂ ON THE NET SYNTHESIS OF CHOLINE IN RATS¹

K. K. GOVIND MENON² AND C. C. LUCAS

Abstract

The base moiety of tissue lecithin appears to arise from dietary components other than choline. Although the presence of choline in the diet is essential for maintaining the health of the animals, supplements of choline do not increase the amount of this base found in the bodies of rats paired-fed purified diets. In contrast, dietary supplements of methionine and of vitamin B₁₂ do increase the total choline content of the carcass of rats.

Introduction

Studies on the biosynthesis of choline, like those on nicotinic acid, are complicated by the interrelationship between the vitamin and an essential amino acid. Tracer studies have shown that the carbon atom of formate, the beta-carbon of serine, the alpha-carbon of glycine, carbon atom 2 of the imidazole portion of histidine, and carbon atom 2 of the indole ring of tryptophan can be incorporated first probably into the methyl group of methionine and then by transmethylation on to ethanolamine or an ethanolamine derivative to form choline. Gradually the roles of vitamin B₁₂ and folic acid in the *de novo* synthesis of choline are being disclosed.

Some effects of diet on the net synthesis of choline have been determined and will be presented briefly. From determinations of the absolute choline contents of the livers and carcasses of weanling rats before and after they had been fed purified diets containing optimal and suboptimal amounts of methionine, estimates of the net increases were made. The influence of vitamin B₁₂ on the accumulation of choline in the body of the rat was also investigated.

Experimental

Materials and Methods

(1) Diet

Because of the interplay of the factors mentioned above, it has been difficult to plan experiments on the biosynthesis of choline that would permit one to draw unambiguous conclusions. The methionine content and the amino acid pattern of the basal diet are factors of utmost importance. Unpublished studies by one of us (C.C.L.) indicated that the methionine requirement of young rats of our colony (in the presence of adequate choline) can vary from about 300 mg to over 420 mg per 100 g of the diet, depending on the cystine content of the ration. For this study a diet (Table I) has been used that is

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Contribution from the Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto 5, Ontario.

²Present address: Department of Pharmacology, Saint Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis 4, Missouri, U.S.A.

TABLE I
Composition of the basal diet used to study the net synthesis of choline

	%		%
Casein	8.0	Starch	10.0
Alpha (soya) protein*	4.0	Dextrin	10.0
Alcohol-extracted peanut meal	4.0	Beef fat	10.0
Salts†	3.0	Corn oil	2.0
Vitamin-sucrose mixture‡	1.0	L-Cystine	0.10
Cellulose	2.0	Cod liver oil conc.‡ (A and D)	0.015
Sucrose	45.9	α -Tocopheryl acetate	0.010

(Protein 14%, methionine 300 mg/100 g, cystine 210 mg/100 g)

* Glidden's Alpha (soya) protein.

† For composition of these mixtures see Best *et al.* (3).

‡ Obtained from Ayerst, McKenna and Harrison Ltd., Montreal. It contains 200,000 I.U. vitamin A and 50,000 I.U. vitamin D/g.

believed to be adequate with respect to all the known vitamins, essential amino acids, and other nutrients (including organic sulphur) but which contains barely sufficient methionine for growth. This amount of methionine (300 mg/100 g) has been shown to be grossly inadequate for lipotropic purposes (1, 2).

Table II shows the supplements that were added to form the various test diets. The average survival time of weanling rats fed the basal hypolipotropic diet (no supplementary methionine, i.e. GVA-0) was only 9 days, for in the absence of choline, fatal haemorrhagic kidney lesions developed. In the presence of the choline supplement (GVA-1), animals survived in apparent

TABLE II
Dietary supplements and their effects on growth

Group No.	No. of rats	Supplements	Average weight at killing
Stock	5	(None; weaned to chow)	40
GVA-0	10	(None; basal diet)	45
GVA-1	6	0.35% choline chloride	62
GVB-0	10	0.1% DL-methionine	60
GVB-1	6	0.1% DL-meth. + 0.35% choline chloride	66
GVC-0	8	0.15% DL-methionine	63
GVC-1	6	0.15% DL-meth. + 0.35% choline chloride	85
GVC-1a*	6	0.15% DL-meth. + 0.35% choline chloride	112
GVK-0	10	0.15% DL-methionine	60
GVK-1	6	0.15% DL-meth. + 0.35% choline chloride	85
GVK-1a*	6	0.15% DL-meth. + 0.35% choline chloride	119
GVK-2	6	0.15% DL-meth. + 10 μ g vitamin B ₁₂ /100 g	83
GVK-2a*	6	0.15% DL-meth. + 10 μ g vitamin B ₁₂ /100 g	98
GVK-3	6	0.15% DL-meth. + 0.35% choline chloride	87
GVK-3a*	6	0.15% DL-meth. + 0.35% choline chloride + 10 μ g vitamin B ₁₂	122

*The rats in these groups were fed ad libitum, those in the others were group-paired fed with the rats of the corresponding group 0.

good health on this diet over the period of study. When 0.1% DL-methionine was added to the basal diet (so that GVB-0 contained 400 mg total methionine per 100 g), the average survival time of the rats was 11 days, and when 0.15% of DL-methionine was added (GVC-0), the animals survived for the experimental period of 3 weeks. The methionine content of this diet (GVC-0) thus appears to be barely sufficient for survival of our strain of rats, when choline is absent, under our experimental conditions.

(2) Animals

Weanling male rats (Wistar strain) reared in our own colony and weighing 40–50 g were used, 6 to 10 per group (Table II). When the rats were being set out randomly in groups, five were killed to give baseline data from which to measure the net formation of choline. The care of the animals and the paired-feeding technique employed have been described by Best *et al.* (3). The diets were fed for 3 weeks or until the negative controls (animals on the deficient rations) died. Whenever a death occurred in the basal group, an animal chosen at random from the corresponding supplemented group was sacrificed.

(3) Methods

The rats were anaesthetized with ether, decapitated, and the liver and kidneys were removed and weighed. The intestinal tract was removed and washed out. The kidneys and intestines were combined with the rest of the body and ground in a Hobart meat grinder, twice with the coarse cutting plate, twice with the medium one. The livers (disintegrated under acetone in a Waring blender) and ground carcasses were extracted individually with hot alcohol, and the lipids soluble in petroleum ether were analyzed (4). Phosphorus was estimated by the method of King (5). Choline was precipitated, after hydrolysis of pooled samples for each group, as enneaiodide according to the procedure of Erickson *et al.* (6) and the optical density of the precipitate dissolved in ethylene dichloride was measured according to the micromethod described by Appleton *et al.* (7). Previous tests had revealed that only a negligible amount of water-soluble derivatives of choline could be detected in the carcasses of normal rats (about 2% of the lipid-soluble form). Water-soluble forms of choline were therefore not determined routinely.

Results and Discussion

As has been observed many times, liver fat increased considerably in the rats fed the choline-free diets (Table III). The body fat was unaffected by choline deficiency. The animals fed ad libitum on diets supplemented with choline, methionine, or vitamin B₁₂ showed slightly higher carcass fat than the corresponding paired-fed animals, due no doubt to the greater food intake and better growth of these animals. The relative amount of carcass lipids showed a tendency to increase as the animals grew: animals whose weights at killing were around 100 g had about 10% fat in the carcass; those weighing around 40–60 g had only 5–8% body fat. In the choline-deficient rats, although there

TABLE III

Total lipid and phospholipid contents of livers and carcasses of rats fed a hypolipotropic diet variously supplemented with lipotropic agents

Group	Average gain in wt. on diet	Hepatic lipids			Carcass lipids		
		Total % liver wt.	P-lipids % liver wt.	P-lipids % liver fat	Total % carc. wt.	P-lipids % carc. wt.	P-lipids % carc. fat
Stock	—	4.6 ±0.26*	2.04	44.0	5.3 ±0.22*	1.82	34.2
GVA-0	5	18.4 ±0.51	1.69	9.2	6.6 ±0.50	1.80	27.2
GVA-1	22	4.9 ±0.21	2.25	46.2	6.3 ±0.56	1.85	29.2
GVB-0	20	14.0 ±0.87	2.41	17.2	8.1 ±0.45	1.66	20.5
GVB-1	26	4.5 ±0.12	2.46	55.1	7.5 ±0.39	1.38	18.4
GVC-0	23	17.0 ±2.46	2.60	15.3	8.2 ±0.86	1.78	21.6
GVC-1	45	5.2 ±0.21	2.47	48.0	9.7 ±0.55	1.86	19.2
GVC-1a†	72	5.3 ±0.21	2.51	47.3	12.9 ±1.85	1.23	9.5
GVK-0	20	15.4 ±1.53	2.74	17.7	8.3 ±0.85	1.48	17.8
GVK-1	45	5.7 ±0.19	2.79	49.2	10.4 ±1.09	1.12	10.8
GVK-1a†	79	5.8 ±0.41	2.90	50.3	9.8 ±0.98	1.09	11.1
GVK-2	43	10.1 ±1.41	3.31	32.7	10.4 ±1.05	1.25	12.1
GVK-2a†	58	8.5 ±1.48	2.89	34.4	10.8 ±1.45	1.52	14.1
GVK-3	47	5.8 ±0.32	3.02	52.4	9.1 ±1.34	1.32	14.5
GVK-3a†	82	5.2 ±0.28	2.79	53.6	12.2 ±0.62	1.24	10.2

*Standard error of the mean.

†These groups were fed ad libitum. All others were paired fed vs. their own control (i.e. the corresponding group 0).

was a considerably increased deposition of glycerides in the liver, there was no corresponding increase or decrease in phospholipids. The *proportion* of the phospholipids in the liver fat is greatly diminished in choline deficiency (8, 9), but it is important to note that the absolute amount is unchanged (4, 8). Choline deficiency did not produce any marked changes in the phospholipid content of the carcass. The percentage of phospholipids in carcass fat showed a tendency to decrease as the animals gained weight. Williams *et al.* (10) have also reported similar effects of age on the total lipids and phospholipid content of the carcass.

Tables IV and V give the data on the net synthesis of choline by the rats during the 3-week experimental period. This was calculated by subtracting from the total body choline of animals at the end of the experiment that found in animals of comparable weight at the start of the experiment.

TABLE IV

Effect of methionine, choline, and vitamin B₁₂ on net choline synthesis (increase in choline content of liver and carcass during 3-week period)

Group	Av. final wt. g (and days on diet)	Amount of choline found (mg)			Formed (exptl. minus stock)
		Liver	Carcass	Total	
Stock	40 (0)	2.84	26.04	28.9	
GVA-0	45 (9)*	7.68	29.29	37.0	8.1*
GVA-1	62 (9)*	6.90	37.42	44.3	15.4*
GVB-0	60 (11)*	11.59	42.15	53.7	24.8*
GVB-1	66 (11)*	8.57	46.71	55.3	26.4*
GVC-0	63 (21)	10.94	56.48	67.4	38.5
GVC-1	85 (21)	7.03	64.83	71.9	43.0
GVC-1a	112 (21)	8.01	75.84	83.9	55.0
GVK-0	60 (21)	10.04	51.20	61.2	32.3
GVK-1	85 (21)	7.08	[49.48 ?]†	[56.6 ?]	[27.7 ?]
GVK-1a	119 (21)	9.69	73.60	83.3	54.4
GVK-2	83 (21)	5.56	68.67	74.2	45.3
GVK-2a	98 (21)	7.08	88.42	95.5	66.6
GVK-3	87 (21)	7.34	67.12	74.5	45.6
GVK-3a	122 (21)	9.87	91.63	101.5	72.6

*It is questionable whether moribund animals dying of acute choline deficiency are strictly comparable with the apparently healthy animals fed the supplemented diet and killed at the same time.

†Some loss suspected during analysis of pooled carcass lipids for choline. GVC-1, a similar group run at an earlier time, contained 64.8 mg choline in the carcass, as compared with the 49.5 mg found in GVK-1. Even if one ignores these results, the effect of vitamin B₁₂ on the synthesis of choline is seen by comparing GVK-2 with GVK-0 (45.3 - 32.3 = 13.0 mg) or GVK-3a with GVK-1a (72.6 - 54.4 = 18.2 mg).

TABLE V

Lack of effect of dietary choline on amount of choline in young rats

Expt.	Total choline (mg) found in body	
	No dietary choline	Plus choline (paired-fed)
GVB	54	55
GVC	67	72
GVK	61	57*
GVK + B ₁₂	74	75
Totals	256	259
Average	64	65

*This value is possibly low; see footnote to Table IV.

The average total choline found in the bodies of the rats fed for 21 days on the diets containing 0.45% total methionine without choline (GVC-0 and GVK-0) was 64 mg; with 0.35% choline chloride present (GVC-1 and GVK-1) the average value was also 64 mg. Under both dietary conditions an increase of (64 minus 29) mg had occurred in 21 days, i.e., a net synthesis of 1.66 mg choline per day. When vitamin B₁₂ was present (GVK-2 and GVK-3) the totals without and with choline in the diet were 74 and 75 mg, respectively. The increase (about 10 mg) in net formation of choline due to vitamin B₁₂ led to a daily increment of 2.14 mg compared with 1.66 mg when B₁₂ was

absent, corresponding to 0.5 mg more per day or about a 30% increase in the net biosynthesis.

That the carcasses of animals fed choline-deficient rations contained essentially as much choline as those of rats that had been paired-fed the same diets supplemented with choline (Tables IV and V) is in agreement with the findings of Jacobi, Baumann, and Meek (11), who first reported that animals dying of choline deficiency contained essentially the same amount of choline as those fed complete diets. Stetten (12) and Boxer and Stetten (13) found that the effect of choline deprivation was to retard markedly the rate of incorporation of new choline into the phospholipids of the body without altering the total quantity of choline present.

It is not surprising that greater amounts of choline were found in the bodies of rats fed *ad libitum*, since these animals with a greater food consumption achieved better growth than did those that were paired-fed.

The fact that in paired-fed animals choline supplements did not produce any significant effect on the total choline content of the body is understandable in view of the findings of Kennedy (14) that free choline is not incorporated into the lecithin molecule. In any case it is doubtful whether there is available more than minute traces, if any, of free choline in metabolically active organs. Increased net synthesis of lecithin is obtained, not when animals are given dietary choline, but when they are given methionine or vitamin B₁₂. Such findings fit in with the hypothesis that body lecithin is formed from precursors other than choline, e.g., phosphatidyl serine, phosphatidyl ethanolamine, or cytidine diphosphoethanolamine. Methionine and vitamin B₁₂ presumably enhance the methylation of these precursors for the formation of lecithin.

It is of interest to mention at this point that Williams,* in our laboratory, has so far been unable to demonstrate choline kinase activity in rat liver preparations. On the other hand, when arsenocholine (15), the triethyl homologue of choline (16), or sulphocholine (17) were fed to rats, all three compounds were found to be incorporated to some extent into liver lecithin. Also, the effect of dietary choline on the rate of appearance of new choline (13) in body lecithin must not be overlooked. Thus, the exact relationship of dietary choline to lecithin formation is still not clear.

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INABILITY OF HETEROLOGOUS ANTIBODIES TO AFFECT RAT THYROID GLAND¹

H. S. SODHI

Abstract

Heterologous rat thyroid antibodies produced in rabbits were injected by intraperitoneal, intravenous, and intra-arterial routes in different groups of rats and the effects on the morphology and 24-hour ¹³¹I uptake of their thyroid glands were investigated. In spite of the administration of high titers of specific antibodies no effects, acute or chronic, were observed, indicating the inability of the heterologous thyroid antibodies to alter the structure or function of the rat thyroid glands.

Introduction

Following the observations that antiserum hemolytic for sheep red cells could be produced by repeated injections of defibrinated blood of sheep into rabbits (1), numerous attempts were made to produce specific cytotoxic antisera for thyroid gland but with no unequivocal results (2, 3, 4). The following work was undertaken to investigate the effects of specific heterologous antibodies on the thyroid gland in vivo.

Methods

Twenty male rats were put on low-iodine diet (Remington) for a period of 2 weeks before they were anesthetized and infused free of blood, and the thyroid glands removed for the preparation of the antigen by the method of Witebsky *et al.* (5). Two rabbits were immunized by repeated intracutaneous injections of the antigen, emulsified in complete Freund's adjuvants, and the antiserum was obtained and stored according to the methods of the above authors.

The antibody titers, when checked by the tanned cell hemagglutination test of Boyden (6), as modified for thyroid (5), were 1:6561 and 1:>19683 in the two specimens of antisera. The antibodies did not react with serum proteins and kidney extract of the rat.

Administration of Antiserum

Thirty female rats which were maintained on ordinary rat purina chow were divided into four groups and treated as follows:

1. Six rats received five intraperitoneal injections of 0.5 ml of antiserum every fourth day and then were sacrificed 4 weeks after the last injection.
2. Six rats received four injections of 0.5–2.0 ml of antiserum in the left carotid artery below the level of the origin of the thyroid artery, and the artery

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Contribution from the Research Laboratory, Jewish General Hospital, and the Department of Investigative Medicine, McGill University, Montreal, Que.

above that level was compressed during the injection. Bleeding was stopped by pressure on the point of puncture. The corresponding part of the opposite artery was compressed for an equal length of time to serve as control. The animals were sacrificed at intervals up to the 23rd day of the first injection.

3. Twelve rats received repeated injections of 0.5 ml of antiserum intravenously and were sacrificed at intervals up to 60 days.

4. Six rats received no treatment and served as controls for group 3. After the sacrifice, the thyroid gland and adjacent trachea were removed and put in Bouin's solution for 24 hours. The tissue was then trimmed and transferred to the tubes containing 2 ml of 70% alcohol for determining their radioactivity prior to the preparation of the sections for histological examination.

Twenty-four hours before the sacrifice, the rats in group 3 (except No. 1 and No. 2) and group 4 (except No. 6) received 2 μ c of I^{131} intraperitoneally for the determination of the 24-hour uptake by the thyroid gland.

The histological sections were stained with hematoxylin and eosin for microscopic examination.

Results

The animals manifested neither acute nor delayed response of any kind to the individual injections of the antiserum, nor were any signs of chronic ill health apparent as a result of repeated administration of the antiserum by the various routes.

The thyroid glands appeared normal to the naked eye in all animals. On microscopic examination, the morphology of the gland in test animals was not different from that of normals. Even the glands of the animals which had repeated compressions of their carotid arteries presented a normal appearance, grossly and microscopically.

The iodine uptake of animals treated with antiserum by intravenous route was not significantly different from that of the untreated controls (Table I). The probability of the difference occurring by chance alone was more than 9%.

TABLE I

Control		Test animals	
Rat No.	% uptake	Rat No.	% uptake
C-1	18.2	3	33.9
C-2	23.0	4	43.8
C-3	40.7	5	27.9
C-4	49.0	6	33.1
C-5	10.8	7	8.9
Mean	29.5	8	34.0
		9	57.5
		10	38.0
		11	25.0
		12	18.9
		Mean	27.1

Discussion

In most of the earlier studies the thyroid proteins probably became denatured during the process of extraction, and were also contaminated by the proteins from the blood as indicated by the generalized hemolytic reaction following the injection of antisera produced in response to those extracts (2, 3, 4). In the present work, the process of extraction was such as to minimize the denaturation of the thyroid proteins and their contamination by blood. The colloid as well as the intracellular contents came out in the extract, as no intact cells could be detected under the microscope in the discarded debris of the tissue.

Thyroglobulin, a potent antigen (7), constitutes about 80% of the total proteins in such an extract (8). Another antigen, intracellular in location, was demonstrated by the differential tanned cell hemagglutination technique, while working with similar extracts from rabbits (9). Two distinct precipitating antigens have been shown to be present in the colloid (10) which do not bind the complement, and one complement-fixing antigen has been demonstrated in the microsomal fraction of thyroid cell homogenates (11); the latter is more abundant in thyrotoxic glands. The rats sacrificed to obtain the thyroid extract were kept on low-iodine diet for 2 weeks, to produce a physical state of their glands similar to that of thyrotoxic glands.

The antiserum was tested only by the tanned cell hemagglutination test and the titers of antibodies were very high. However, there is no reason to believe that complement-fixing and other antibodies were not produced simultaneously in response to the other antigens present in the thyroid extract. The administration of this antiserum failed to produce any morphologic changes even when high concentrations of specific antibodies were made available to the thyroid gland, indicating the inability of these antibodies to initiate and continue pathological changes in the gland. The work of the group in England (12, 13) does suggest the presence of a cytotoxic factor distinct from the known antibodies, which is actually responsible for the damage to cells. The nature of this factor and its relation to the various known thyroid antigens and antibodies is yet unknown.

Subsequent to immunization with human thyroglobulin, precipitating antibodies appeared in the sera of rabbits and these animals developed changes suggestive of hypofunction of the thyroid, but no morphologic changes of the glands were found on microscopic examination (14).

Thus, radioiodine studies were done in the present work to find out if the function of thyroid gland could be altered by heterologous circulating antibodies without effecting its microscopic appearance. No disturbance in the 24-hour iodine uptake by the thyroid gland was noted in the test group injected intravenously with repeated doses of specific antithyroid serum.

Conclusion

Antibodies produced in rabbits following injection of saline extract of the rat thyroid though specific for thyroid are not cytotoxic to the thyroid gland *in vivo*.

Acknowledgments

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THE ASSAY OF INSULIN WITH ANTI-INSULIN AND MOUSE DIAPHRAGM¹

A. C. WARDLAW AND P. J. MOLONEY

Abstract

A method is described for assaying insulin in which anti-insulin is used as an insulin-neutralizing reagent and glycogen synthesis by mouse diaphragm as an indicator of free insulin. The test is thus analogous to the titration of a toxin with an antitoxin, using a biological indicator to determine the end point. The effect of using antiserum in the diaphragm test is to increase the slope of the dose-response curve about 30-fold, thus giving a test system which is capable of high precision with relatively few observations. This is reflected in the low λ -value of 0.015 for the diaphragm test with antiserum compared with 0.45 for the test without antiserum. Data are presented showing that the immunological test has good reproducibility and is capable of measuring the potency of unknown insulins with acceptable precision using only small numbers of animals. Sixteen samples of insulin in various stages of purification from seven mammalian species gave results by the new method in close agreement with mouse convulsion data. Two samples of fish insulin were found to be immunologically different from mammalian insulin and therefore could not be assayed with anti-ox-insulin sera.

Introduction

One of the drawbacks of the standard rabbit and mouse methods for assaying insulin is the large number of observations which are needed to obtain results of acceptable accuracy. This has stimulated much research into alternative methods for assaying insulin, and a considerable number of chemical (1), physiological (2, 3, 4, 5), and immunochemical (6, 7) assay techniques have now been described. In general, however, these methods either offer little advantage in precision over the standard methods, although they may be much more sensitive, or, they do not actually measure hormonal activity which is a disadvantage from the viewpoint of the biological standardization of insulin preparations. Furthermore, the validity of the alternative methods has not, in general, been checked by making parallel tests with the standard methods to see if good agreement can be obtained with insulins in various states of purity and from different animal species.

The insulin assay described here is a biological method which has good sensitivity, and is capable of high precision with relatively small numbers of animals. The method is similar to the titration of a toxin with an antitoxin, using a biological indicator to detect the end point, and it depends for its precision on the same principle as that which operates in such tests, namely, that the slope of the toxin dose-response curve can be greatly increased if the dilutions of toxin are mixed with a constant dose of antitoxin before injection into the test animals. The higher the level of antitoxin, in terms of minimum

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Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

lethal doses (M.L.D.'s) neutralized, the steeper the slope. For example, if the level of antitoxin used per injected dose is sufficient to neutralize 100 M.L.D. of toxin, then a small percentage change in toxin concentration in the region of 100 M.L.D. will cause a change from zero to maximum in the toxicity of the final mixture. Thus the use of antitoxin imparts great resolving power to the test and enables closely spaced dilutions of toxin to be differentiated with only small numbers of animals at each dose. It should be noted that the usefulness of the test may be lessened if the solution of toxin contains appreciable quantities of toxoid, a complication which does not arise with insulin.

The possibility of applying this procedure to the assay of insulin developed from previous work in these laboratories on the preparation of antisera capable of neutralizing the hormonal activity of insulin (8, 9, 10). In using these antisera for insulin assay, it was desirable that the biological indicator for free insulin should be of high sensitivity so that only small amounts of serum would be consumed in each test. Use was made, therefore, of the rat diaphragm test, whose applicability in studying the neutralization of insulin by anti-insulin has already been reported by Wright (11). This author took, as indicator of an insulin effect, the augmented uptake of glucose by the tissues. We have used the mouse instead of the rat and have taken augmented synthesis of glycogen by the tissues as the measure of insulin activity.

This paper is divided into two sections. The first describes the technique and some features of the mouse hemidiaphragm - glycogen test without antiserum. The second deals with the hemidiaphragm test as an indicator for the neutralization of insulin by anti-insulin and the application of this system to the assay of insulins in various stages of purification and from several animal species. Data are presented on the reproducibility of the neutralization test, and on its fidelity as shown by assays on insulin samples of known potency but tested under code. Where necessary, proper controls have been made by a standard mouse convulsion method.

Materials

Mice

Male white mice of the Connaught strain were used. They were maintained on a diet of pellet food ("fox cubes": Master Feed Co., Toronto).

Media

The fluid used for incubating tissues was the Gey and Gey balanced-salts-bicarbonate medium (12) containing 3 mg/ml glucose, 1 mg/ml gelatin, and 3 μ g/ml phenol red and having a pH of 7.2 when gassed with a mixture of 95% O₂ + 5% CO₂. For convenience, the complete medium was prepared as two partial media, medium A and medium B, and, in addition, a medium lacking glucose, referred to as "soaking medium" was used for soaking the tissues prior to incubation. The media were compounded by mixing the stock solutions described below in the proportions set out in Table I and were stored in 50-ml amounts at -25° C. The compositions of the stock solutions were as follows:

TABLE I
Composition of media

Stock solution (described in text)	Medium A (ml)	Medium B (ml)	Soaking medium (ml)
Gey and Gey basal salts	200	100	100
Gey and Gey bicarbonate 10×	0	50	25
Gey and Gey phosphate 10×	50	25	25
3 mg/ml soluble phenol red	0	0.5	0.25
10% w/v gelatin	5	2.5	0
30% w/v glucose	0	5	0
Distilled water	To 500	To 250	To 250

NOTE: The pH of medium A is 7.2 and does not require gassing with 95% O₂ + 5% CO₂. Gelatin is added to minimize possible adsorption of insulin to glassware (13, 14).

Gey and Gey basal salts consisted of 28.55 g NaCl, 1.065 g KCl, 0.75 g CaCl₂, 1.32 g MgCl₂·6H₂O, and 0.2615 g K₂SO₄ dissolved to 2 liters in distilled water. Gey and Gey phosphates 10× consisted of 0.1705 g KH₂PO₄ and 0.855 g K₂HPO₄·3H₂O dissolved to 500 ml in distilled water. Gey and Gey bicarbonate 10× consisted of 11.45 g NaHCO₃ dissolved to 500 ml in distilled water. Phenol red stock solution consisted of 0.3 g soluble phenol red in 100 ml distilled water. Gelatin 10% was prepared by dissolving 10 g purified calfskin gelatin (Eastman) in hot water, adjusting to pH 7.2 with 30% NaOH, making the volume to 100 ml and filtering through paper at 37° C. Glucose was prepared as a 30% w/v stock solution in distilled water. The glucose and gelatin were stored frozen at -25° C; the other components were kept at room temperature.

Anthrone Reagent

This consisted of 0.5 g anthrone (Fisher reagent) dissolved in 500 ml 84% v/v sulphuric acid. It was filtered through glass wool, stored at +5° C, and used within 4 days.

Insulins

Samples of insulin from ox, pig, monkey, horse, sheep, whale, mouse, catfish, codfish in various stages of purification, and also insulins regenerated from fibrils, were obtained from these laboratories. Standard insulin was prepared in a final concentration of 20 units/ml in the diluent described in the United States Pharmacopoeia (XV edition).

Anti-insulin Sera

These were obtained from guinea pigs and from horses by methods already described (8, 9, 10). The sera were distributed in 2-ml amounts in tubes and stored at -25°. When required, a tube of serum was melted and kept at 1° until used up.

Methods

The tests were carried out in flat-bottomed 1×4 in. Pyrex tubes made by cutting down and flattening ordinary boiling tubes. The empty tubes were gassed with 95% O₂ + 5% CO₂, stoppered, and placed in an ice bath. Then

0.8 ml of well-gassed medium B was added to each tube with a 1-ml serological pipette and this was followed by a volume of medium A sufficient to give a total volume 1.6 ml after the other reagents had been added (e.g. insulin, anti-insulin). If antiserum was to be used, it was added to the mixture of media A and B in volumes ranging from 0.01 to 0.1 ml using an Agla micrometer syringe (Burroughs-Wellcome, Beckenham, England). The syringe was clamped horizontally and was fitted with a $\frac{1}{2}$ in., 26-gauge needle from which the tip had been removed and which had been bent through an angle of 90° . The needle was dipped into the mixture of media A and B while making delivery. Care was taken to bring the serum to room temperature before filling the syringe so that formation of air bubbles within the syringe was avoided. Doses of standard insulin, usually 0.01 to 0.05 ml of a 1.0 unit/ml solution, were then added from a second Agla syringe. The mixtures of insulin and anti-insulin were well mixed and allowed to remain in the ice bath for $\frac{1}{2}$ to 1 hour before the mouse hemidiaphragms were added. Control experiments showed that, in fact, the insulin-anti-insulin reaction was complete within a few minutes. Hemidiaphragms were dissected and soaked as described below and transferred in groups of three or four to the test mixtures. The tubes were regassed until the color of the fluid was pale orange and then they were tightly stoppered. The tubes were shaken (120 strokes/minute) for 90 minutes in a water bath at 37°C , then cooled in an ice bath and the hemidiaphragms taken out, washed, and analyzed individually for glycogen.

Treatment of Mice and Dissection of Hemidiaphragms

At 5 p.m. on the day before a test, mice in groups of 10 within a weight range of ± 0.5 g were placed in cages without food, but with water ad libitum, until 11 a.m. when tests were usually begun. The animals were killed by cervical dislocation followed by decapitation and the blood was allowed to drain. The dead mice in groups of four were then pinned out on a dissection board and the hemidiaphragms were removed. In practice it was found convenient for one operator to kill the mice and open the peritoneal cavity while a second operator removed the hemidiaphragms.

Hemidiaphragms were removed as follows. The xiphoid cartilage was grasped with artery forceps which were locked at right angles to the mouse and twisted half a turn so as to depress the rib cage and expose the diaphragm. The diaphragm was then punctured with sharp forceps in the central non-muscular area between the inferior vena cava and the xiphoid process. This releases the negative pressure in the chest and causes the diaphragm to billow out. The diaphragm was gripped with forceps at the place of puncturing, and the right and left hemidiaphragms were dissected out with half-inch straight scissors. With each hemidiaphragm, the first cut was made from ventral to dorsal, close to, but not cutting, the inferior vena cava. Without releasing the grip on the tissue, the severance was completed by cutting around the margin of the rib cage. The dissected tissues were transferred without delay into 40 ml of soaking medium continuously gassed with $\text{O}_2 + \text{CO}_2$ and cooled in an ice bath. Gassing

was continued for 10 minutes, after all the tissues had been collected, the rate being sufficient to keep the tissues in motion. The tissues were transferred in groups of three or four to the incubation tubes containing the test solutions which were then incubated as described above (during this time the color of the mixture must remain pale orange).

Estimation of Glycogen

The method used was, with minor modifications, that described by Seifter *et al.* (15). The estimations were made in 6 in. \times $\frac{5}{8}$ in. test tubes which had previously been matched for optical equivalence. Each analysis tube had a permanent marking on it at the 8-ml level and an identifying number at the top. The tissues from each incubation vessel were washed with distilled water on a Hirsch funnel and drained. Each hemidiaphragm was transferred with forceps to a separate analysis tube containing 1.0 ml 30% w/v KOH. When all samples had been collected, the rack of tubes was immersed for 20 minutes with periodic shaking in a bath of boiling water, and finally cooled in ice water. Next, 1.25 ml of 95% ethanol was added to each tube from an automatic measuring syringe, the tubes shaken and allowed to stand for at least 15 minutes prior to centrifugation (30 minutes, 1500 r.p.m.). The supernatants were then poured off and the tubes left inverted to drain over a pad of glass wool for about half a minute. The glycogen residues were dissolved in 2 ml water which was added from an automatic measuring syringe and then each tube was filled up to its 8-ml mark with ice-cold anthrone reagent delivered from a separating funnel. The tube contents were thoroughly mixed in a Vortex mixer (Scientific Industries Inc., Springfield, Mass.) and the rack of tubes was placed in the boiling-water bath for 10 minutes, and cooled immediately in the ice bath. The optical density (O.D.) of each tube was then read in a Coleman colorimeter with a red filter having maximum transmission at 650 m μ , which is close to the $E_{\max} = 620$ m μ of the blue color developed. The instrument was standardized against a blank consisting of 2 ml water + 6 ml anthrone reagent heated in the rack with the samples.

In some tests, known glucose solutions were run in parallel with the samples so that the optical density readings could be expressed as "micrograms glucose". This was not done routinely, however, since it was only the increase in glycogen due to insulin which was of interest, rather than the absolute amount. Also in early experiments it was desired to know the dry weights of the hemidiaphragms so that the glycogen value could be expressed as per unit weight of tissue. This was done by drying the tissues, after incubation, on aluminum foil in an oven at 105° for 2 hours. After being weighed, the tissues were dropped into the KOH in the analysis tubes and the glycogen estimation was done as described.

Mouse Convulsion Test

Mouse convulsion tests were done by the cylinder-cage method of Young and Lewis (16) using 36 mice per cylinder-cage. Potency ratios and confidence limits were calculated by the quantal response method using angular transformations.

Results

THE MOUSE HEMIDIAPHRAGM TEST WITHOUT ANTI-INSULIN

Preliminary Experiments

At the outset of these studies, the effect of insulin on tissue metabolism was investigated with the rat as the source of diaphragm tissue and glucose disappearance from the medium as the metabolic indicator. Results were expressed as micrograms glucose consumed per milligram dry weight of tissue per hour, and insulin activity was detected as an increase in glucose consumption above the basal level. Since the object of this section of the work was to simplify as much as possible the procedure for detecting insulin *in vitro*, one of the first modifications studied was the use of the mouse in place of the more expensive and less easily handled rat. Tests showed the mouse and rat to be equally satisfactory as sources of diaphragm tissue, and the smaller size of the mouse did not present any manipulative difficulties in dissecting the tissues; indeed it was an advantage in handling and killing the animals. The mouse was therefore adopted in place of the rat. Another divergence from the initial technique was the use of glycogen synthesis rather than glucose uptake as the metabolic indicator. Technically, the glycogen analysis is as simple to perform as the Somogyi-Nelson test for glucose and requires no pipetting operations for individual samples since the entire hemidiaphragm is analyzed for glycogen. Furthermore the percentage difference in the amount of glycogen synthesized in the presence and absence of insulin is much greater than the corresponding difference in final levels of glucose in the media. In a typical experiment, for example, the glucose level in the control tube fell to 95% of initial concentration and in the insulin tube to 90%, that is, a difference of approximately 5% in the estimated values for glucose, whereas the anthrone readings (O.D.) for glycogen were 0.180 and 0.360, i.e. a difference of 100%. Hence a given percentage error in the determination of glucose will have a much greater effect on the final result than the same error in the determination of glycogen.

The difference between the amount of glycogen in the insulin tube and the control tube expressed as a percentage is referred to as the "insulin effect".

One of the time-consuming features of the initial procedure was determining the dry weights of the tissues after incubation. However, experience showed that provided the mice were within a weight range of ± 0.5 g and provided a uniform dissection technique was used, it was not necessary to correct for variations in the weight of individual hemidiaphragms, since the correction for weight was small in comparison to the biological variation of the tissues.

Factors Affecting the Sensitivity and Reproducibility of the Test

Mouse Weight

In order to determine the best size of mouse for this work a series of experiments was made in which tissues from altogether 120 mice of weights between 12 and 34 g were tested for responsiveness to 1 milliunit/ml insulin. It was found that although the insulin effects were very variable, there was a definite

and statistically significant ($P < 0.01$) tendency for the tissues from the smaller animals to give a greater insulin effect. Thus whereas 12-g mice gave a mean insulin effect of 60% above the base line, 20-g mice gave a response of 50% above base line, 30-g mice a value of 36%, and 34-g mice, 32%. However, the 12-g mice were difficult to dissect and it was decided that animals weighing close to 20 g offered a satisfactory compromise between responsiveness to insulin and technical convenience.

Effect of Soaking the Tissues before Incubation

It is common in work with rat diaphragms to place the tissues, immediately after dissection, into either saline or Gey and Gey medium which has no glucose or insulin. The tissues are allowed to soak at 0° for 10–15 minutes before being transferred to the glucose and insulin media for incubation. To see whether soaking affected the tissues in any way, a direct comparison was made of soaked and unsoaked tissues from the same groups of animals. Both types of tissues were placed directly in the incubation vessels after dissection, the soaked tissues were given prior treatment in soaking medium for 10 minutes at 0°. To minimize variation, each group of four hemidiaphragms was made up of two left and two right hemidiaphragms from four mice. The tissues were then incubated and analyzed in the usual way. The insulin effect with the unsoaked tissues was $39\% \pm 6$ and with the soaked tissues was $47\% \pm 5$. Although the difference was not statistically significant the results suggest that soaking the tissues made them more responsive to insulin. Other experiments provided similar data, and it was concluded that soaking certainly did not decrease the responsiveness of the tissues and probably increased it. It was found that tissues could be kept for 30 to 45 minutes in soaking medium at 0° and still show a good response to insulin. It became normal practice in this work to dissect the tissues from all the animals, up to 16, into a common vessel of soaking medium, which was gassed for 10 minutes after the last hemidiaphragm had been dissected.

The Use of Quarter-Diaphragms instead of Hemidiaphragms

The amount of glycogen present in a hemidiaphragm after incubation is considerably more than is needed for the glycogen analysis. Experiments on the same pattern as the soaking experiments were therefore made to see if hemidiaphragms could be divided further and still prove satisfactory. Quarter-diaphragms were prepared by cutting the hemidiaphragms transversely, and their metabolic activity compared with that of hemidiaphragms. The mean relative anthrone readings and standard errors were 359 ± 15 and 219 ± 7 for the hemidiaphragms with and without insulin, and 389 ± 23 and 227 ± 17 for the quarter-diaphragms with and without insulin. The quarter-diaphragms did not show a significantly greater insulin effect than hemidiaphragms although they did show significantly ($P = 0.05$) more variation. It appeared that there was no real advantage in using the quarter-diaphragms.

Effect of Incubation Time

It is normal practice in glucose-uptake studies with rat diaphragm to use a 1- to 2-hour incubation period before stopping the reaction for analysis. With mouse diaphragm it was found that maximum divergence between amounts of glycogen synthesized in the insulin and control tubes was reached in approximately 90 minutes.

Response of Hemidiaphragms to Different Levels of Insulin

The sensitivity of mouse hemidiaphragms to insulin was investigated by incubating the tissues in different concentrations of insulin over a wide range from 0.05 to 50 milliunits/ml. The data, plotted as a dose-response curve, are shown in Fig. 1. It will be noted that the slope is small and the degree of scatter of the points is relatively high. The lowest level of insulin which gave a definite effect was about 0.1 milliunit/ml although this varied from day to day with different batches of hemidiaphragms. A good insulin effect was always obtained with 1 milliunit/ml but this effect increased only slightly when the insulin concentration was increased to 10 milliunits/ml. Fifty milliunits per milliliter gave no better response than 10 milliunits/ml. Thus the useful part of the dose-response curve is the region from 0.15 to 1.0 milliunit/ml. These concentrations refer to the final concentration of insulin in the 1.6 ml of medium.

To calculate the slope of the dose-response curve, the ordinate was transformed to optical density (O.D.) readings by dividing the ordinate numeral in Fig. 1 by 10. The abscissa was expressed as \log_{10} dose and the slope, measured in the central, steepest portion of the curve, was calculated as $\Delta\text{O.D.}$ divided by $\Delta\log_{10}$ insulin concentration. In Fig. 1, a 10-fold increment in dose produced

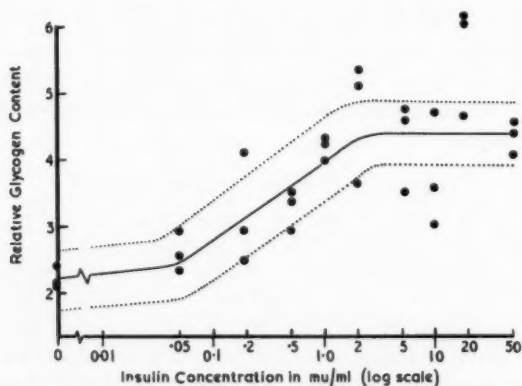


FIG. 1. Response of hemidiaphragms to different concentrations of insulin. Each point represents one hemidiaphragm, and the relative glycogen content is the optical density reading in the anthrone test multiplied by 10.

an optical density increase of 0.11 which is the value for the slope. The standard deviation is 0.05 (on the optical density scale) which is about one-quarter of the

difference between the maximum and minimum responses. The precision of the dose-response curve ($\lambda = \text{S.D.} \div \text{slope}$) is 0.45.

THE MOUSE HEMIDIAPHRAGM TEST WITH ANTI-INSULIN

Potency of Anti-insulin Sera

Prior to carrying out an immunological assay of insulin it was first necessary to establish the neutralizing potency of the antiserum to be employed. This was conveniently done in two stages. First, a preliminary test was made using twofold dilutions of the serum and constant amounts of standard insulin. An example of such a test is shown in Table II. The results in the table show that the potency of the serum lay between 400 and 800 milliunits/ml.

TABLE II
Example of a preliminary titration of an anti-insulin serum
by the hemidiaphragm method

Tube	Test mixture (ml)				Relative glycogen content of hemidiaphragms*	Mean glycogen	Conclusion
	Med. B	Med. A	Anti-insulin	1 unit/ml insulin			
1	0.8	0.68	0.1	0.02	2.20	2.08	Excess antibody
					2.20		
					1.85		
2	0.8	0.73	0.05	0.02	1.80	1.87	Excess antibody
					2.00		
					1.80		
3	0.8	0.755	0.025	0.02	4.60	5.28	Excess insulin
					6.20		
					5.05		
4	0.8	0.76	0.0125	0.02	6.55	6.20	Excess insulin
					6.40		
					5.65		
5	0.8	0.78	0	0.02	4.90	5.40	Insulin control satisfactory
					6.30		
					5.00		
6	0.8	0.80	0	0	2.20	2.16	No-insulin control satisfactory
					2.00		
					2.30		

NOTE: The potency of antiserum is expressed in terms of units of insulin neutralized.
CALCULATION: The volume of anti-insulin serum required to neutralize 20 milliunits of insulin is between 0.05 and 0.025 ml. Therefore the neutralizing potency of the undiluted serum is between 400 and 800 milliunits/ml.
**E₅₀₀* Anthrone readings multiplied by 10.

A more accurate assay was then carried out in which the serum level was kept constant and the insulin level increased by increments of 10 to 20%. Figure 2 shows such a typical standardization curve of glycogen content of hemidiaphragms versus dose of standard insulin added to constant anti-insulin. The composition of the mixtures was similar to those in Table II except that the constant volume of antiserum was 0.03 ml in each tube and the level of insulin was increased by increments of 17% from the lowest level which was 11 milliunits per mixture of total volume 1.6 ml. It is apparent that the dose-

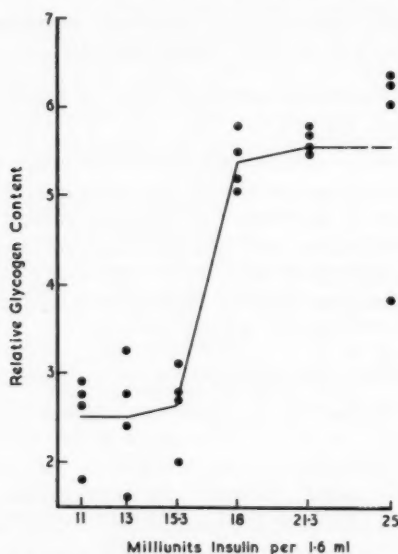


FIG. 2. Response of hemidiaphragms to mixtures of different concentrations of insulin and a constant concentration of anti-insulin. Each point represents one hemidiaphragm, and the relative glycogen content is the optical density reading in the anthrone test multiplied by 10.

response curve has three distinct portions. The three doses on the left (Fig. 2) are in the neutralized area where there was excess antibody, while the three levels on the right are in the region of steep slope where a 17% increment in dose produced a change from zero to maximum response. The slope calculated as described above is 4.1, an increase of 37-fold over the slope of the hemidiaphragm test without antiserum. General experience has indicated that this value 4.1 is probably close to the maximum value attainable in this system, and was observed in this particular experiment because of choice of suitable dose levels. It is apparent, for example, that if the 15.3 dose had not been set up, then the curve would have been drawn between the responses at 13 and 18 milliunits. This would give a slope of 2.05 which is in the range of values usually observed. Thus in other experiments where slopes lower than 4.1 are reported, this is due in part to having used test doses which were too widely spaced.

The standard deviation of hemidiaphragm responses within doses in Fig. 2, is 0.06 on the optical density scale and this gives a λ -value of 0.015, which is average for the system.

Level of Anti-insulin per Test

It might be expected, from analogy with other systems, that in these neutralization tests, the slope of the dose-response curve would increase as the level of

antibody per test increased. To explore this matter, experiments were made in which anti-insulin was used at three different levels of 10, 20, and 60 milliunits per tube. At each level, a three-dose titration was set up using different levels of insulin at the same relative dilution spacing in each titration. Each of the nine insulin-anti-insulin mixtures was made in duplicate and three hemidiaphragms were added to each. The results are given in Fig. 3 as three scatter

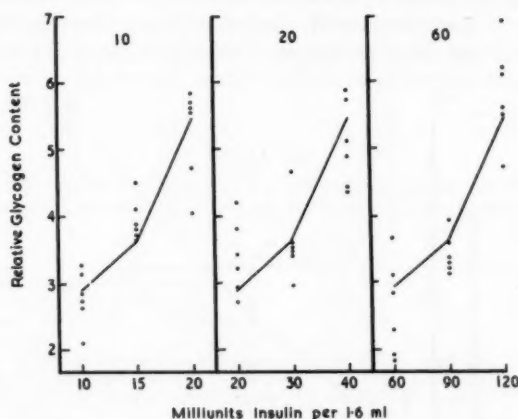


FIG. 3. The effect of concentration of anti-insulin on the slope of the dose-response curve. The figures 10, 20, and 60 refer to the number of milliunits of anti-insulin in each 1.6-ml test mixture. Each point represents one hemidiaphragm, and the relative glycogen content is the optical density reading in the anthrone test multiplied by 10.

diagrams of glycogen content of individual hemidiaphragms plotted against the insulin level in each tube. From the over-all data a mean dose-response curve was calculated and superimposed in an identical manner on each of the three scatter diagrams. Inspection of Fig. 3 suggests that there is no significant tendency for the slope to increase as the level of antiserum is increased from 10 to 60 milliunits per tube. From the practical viewpoint this means that satisfactory neutralization tests can be run at the economical antiserum level of 10 milliunits per tube. In other similar experiments where a comparison was made between using 2 milliunits anti-insulin per tube and 20, it was found that the lower level definitely gave a less steep slope.

Assays of Unknown Insulin against Standard Insulin

The method used to assay an unknown insulin consisted in adding serial dilutions of it to constant amounts of antiserum and comparing the hemidiaphragm responses with those given by known amounts of standard insulin added to the same anti-insulin and tested with the same batch of tissues. The assay was thus based on the direct comparison of sample insulin with standard insulin rather than with the anti-insulin itself. In the case of a completely unknown sample, the procedure was to make a series of 5-fold or 10-fold dilu-

tions (0.75-ml volumes) in medium A, add constant amounts (0.05 ml) of anti-insulin (in the range 10–25 milliunits), and determine which mixtures contained free insulin and which had excess antibody. This gave a rough estimate of potency, from which closer and closer estimates could be obtained by making tests with two-fold and then with 15%– to 20%-spaced dilutions of sample. With a totally unknown sample, it was usually found most convenient to obtain the rough estimate of potency by mouse convulsion test, using groups of 12–24 animals at each dose level, and then apply the diaphragm test. An example of a titration using 17%-spaced dilutions is given in Fig. 4 where three

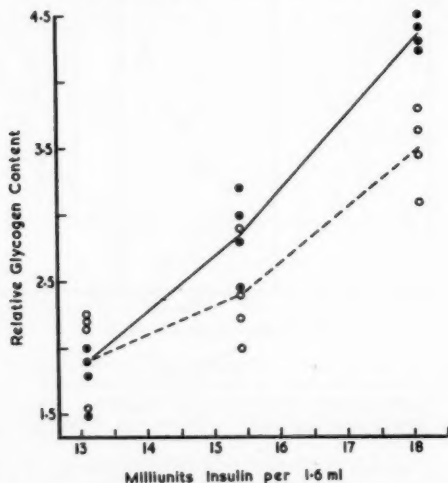


FIG. 4. Assay of an unknown insulin (open circles) against standard insulin (closed circles). Each point represents one hemidiaphragm, and the relative glycogen content is the optical density reading in the anthrone test multiplied by 10.

doses of standard insulin and three of unknown were run in parallel against a constant level of antiserum. The "unknown" in this case was an ox insulin whose potency had been determined to within $\pm 20\%$ by the mouse convulsion test. On the assumption that the convulsion figure was correct, the sample was diluted to 1 unit/ml and then run at the same three doses as the 1 unit/ml standard, namely, 13, 15.4, and 18.2 milliunits per incubation mixture. It is apparent from Fig. 4 that the sample was somewhat less potent than the standard. Interpolation of the 15.4- and 18.2-sample responses on to the standard curve gives estimates of 0.932 and 0.915 (mean = 0.924) for the potency ratio of unknown to standard.

Ninety-five per cent confidence limits of approximately $\pm 4\%$ were obtained by calculation of the over-all standard deviation of hemidiaphragm responses within mixtures and by interpolation on the curve given by the standard insulin with anti-insulin.

Reproducibility of the test.—In order to test the reproducibility of the assay procedure, experiments were made in which a single insulin sample was titrated repeatedly in a two-dose test using three hemidiaphragms per dose. The titrations were done on 4 days, and altogether 18 replicate two-dose tests were made. From the summary of the results given in Table III it is apparent that the test is capable of a high degree of reproducibility. For purposes of presentation, one of the replicate tests on each day was arbitrarily chosen as "standard" and the other tests designated as "unknowns". The potencies of the "unknowns" were then calculated as a percentage of the potency of the "standard". The standard deviation of potency estimates within experiments was 2.2% and the mean λ -value 0.015.

TABLE III

Reproducibility of the dose-response curve of the hemidiaphragm test with antiserum

	Expt. A	Expt. B	Expt. C	Expt. D
Number of replicate 2-dose tests in expt.	3	5	5	5
Total no. of hemidiaphragms used in expt.	18	30	30	30
Mean optical density (O.D.) given by tissues exposed to high dose	0.356	0.361	0.448	0.440
Mean O.D. given by tissues exposed to low dose	0.230	0.270	0.332	0.315
Mean slope = $(\Delta O.D.)/(\Delta \log_{10} \text{dose})$	2.235	1.625	2.075	2.230
Standard deviation within groups of three tissues (O.D. units)	0.022	0.025	0.037	0.034
λ -value	0.011	0.015	0.018	0.015
Potency of "unknowns" as a percentage of "standard"	(1) 98.5 (2) 99.0 (3) — (4) —	96.0 97.5 98.0 100.0	100.0 100.0 102.0 100.0	102.0 103.0 100.0 98.5

NOTE: Each experiment, A, B, C, D, was done on a different day using low and high doses of 21.5 and 24.5 milli-units of insulin per tube and constant amounts (0.03 ml) of anti-insulin.
Mean λ = 0.015; mean slope = 2.04; mean standard deviation within groups of three tissues = 0.029; standard deviation of potency estimates within experiments = 2.2%.

Assay of Known Insulins Tested under Code

The above experiments although giving information on the variation between replicate assays on the same sample do not provide direct evidence on how accurately an unknown insulin may be assayed. Since this is the situation encountered in working practice, it was decided to investigate it directly by titrating samples of known potency but tested under code. Arrangements were therefore made for an external referee to prepare accurate volumetric dilutions of a standard insulin and to send them for test labelled A, B, C, D, etc. It was agreed that the potencies of the samples would be within the range 70% to 130% of a standard supplied by the referee, but the choice of the actual values was left to the referee. The assays were performed as described above using constant anti-insulin and suitable dilutions of standard and unknown. On each day, the standard was set up at two or three doses to span the steep part of the dose-response curve, as in Fig. 4, and four hemidiaphragms were used at each dose. The unknowns were tested by assuming potencies of 85%,

95%, 105%, and 115% of standard and, on this basis, by using amounts which would give responses intercepting the center of the dose-response curve of the standard. Either two or three hemidiaphragms were used per dose in these preliminary tests, which usually fixed the potency to within a $\pm 10\%$ range. After these tests were made, each sample was run once or twice more, using closely spaced dilutions in an appropriate range, to obtain responses which intercepted the standard curve. When all 18 samples had been tested, the code was broken and a comparison made between the true potencies and the estimated values. It is apparent from Table IV that, in general, there was very

TABLE IV
Results of assays by the hemidiaphragm test with antiserum
on insulin samples of known potency

Sample	True potency (units/ml)	Estimate of potency (units/ml)	Deviation from true potency (%)
A	1.14	1.13	-1
B	0.78	0.78	0
C	0.90	1.07	+19
D	0.90	0.95	+6
E	0.78	0.83	+6
F	1.14	1.06	-7
G	0.72	0.695	-3
H	0.72	0.72	0
I	1.08	1.115	+3
J	1.08	1.14	+5.5
K	0.96	1.01	+5.5
L	0.96	1.05	+9.5
M	1.20	1.27	+6
N	1.02	1.13	+11
O	1.02	1.05	+3
P	0.84	0.86	+2
Q	1.20	1.05	-12.5
R	0.84	0.79	-6

NOTE: The standard deviation of the difference between the estimated potencies and the true values = 7%.

The samples were tested under code which was not broken until the tests were completed.

close agreement between the estimates and the true values. Thirteen of the eighteen values were within 6% of the true values, and of the remaining five, three were within 7-11% and two, samples C and Q, showed the rather large discrepancies of 19% and 12.5% respectively. The over-all standard deviation of the difference between the estimated and the true potencies is 7%.

Assays on Different Kinds of Insulin

Insulins in different stages of purification and from a variety of mammals and fishes were assayed against an ox insulin standard. The same samples were also assayed by the mouse convulsion method so that results by the two tests could be compared. The results in Table V on 16 samples from nine species show that, with mammalian insulins, there is a good correlation between the diaphragm test and the convulsion test within the limits of their respective

TABLE V

The potencies of various insulins measured by the hemidiaphragm test with antiserum and by the mouse convulsion test

Description of insulin	Mouse convulsion test (units/mg (except for* values which are units/ml) ± 95% confidence limits)	Hemidiaphragm test with antiserum (units/ml)
Ox, crude mother liquors from crystallization	0.83 ± 20%	0.98 ± 12%
Ox, crude extract of pancreas	12* ± 20%	11* ± 20%
Ox, amorphous	14 ± 19%	13 ± 10%
Ox, crystalline 932	23.4 ± <20%	22.5 ± 10%
Ox, crystalline, regenerated from fibrils	26* ± <25%	23* ± <20%
Pig, crude extract of pancreas	18* ± <25%	20* ± 10%
Pig, amorphous	18 ± 13%	20 ± 10%
Pig, crystalline	23.3 ± 35%	24 ± 10%
Pig, crystalline, regenerated from fibrils	21 ± 25%	21 ± 10%
Sheep, crude	0.05 ± <25%	0.047 ± 10%
Horse, purified	19.6 ± 14%	20.3 ± <20%
Monkey (<i>Macaca mulata</i>), purified	21.3 ± 11%	25 ± <20%
Whale (<i>Physeter catodon</i>), purified	22.5 ± 12%	28 ± <25%
Mouse, crude	0.06 ± 50%	0.05 ± 10%
Codfish, crystalline	12* ± <25%	> 200*
Lake catfish, crude	0.3* ± <25%	> 3.0*

errors. With the two fish insulins, on the other hand, there are marked discrepancies between the potencies obtained by the two methods, the diaphragm values being much higher. This result is due to a marked difference in the specificity of the mammalian and fish insulins in question. Presumably, if suitable antisera against the fish insulins were available, then these samples could be titrated satisfactorily against fish insulin standards.

Discussion

The method for the assay of insulin which is described here has certain advantages over other methods. For example, the standard whole-animal methods, although they have the merit of measuring hormone activity, are relatively insensitive and also involve the maintenance of large numbers of animals which must be used because of biological variation. Variation is also a drawback of the various tissue methods for assaying insulin in vitro, although these methods have a definite advantage in sensitivity over the whole-animal procedures. The only methods in which variation is small are certain chemical and immunochemical methods. However, these procedures, although they may be sensitive, do not necessarily measure hormone activity.

In the method described here the conventional diaphragm test was modified in the following way: the mouse was used instead of the rat; glycogen synthesis was measured instead of glucose uptake; weighing of tissues was omitted; and anti-insulin serum was used as a specific agent for neutralizing insulin. None of these devices is original, although they do not appear to have been used together elsewhere. Thus Oyama and Grant (5) have recently reported an assay pro-

cedure using mouse hemidiaphragm and glucose uptake, while Gemmill, in one of the original papers (17) on the rat diaphragm test, measured both glucose uptake and glycogen synthesis. In the present investigation, glycogen analyses were done by a modification of the anthrone method of Seifter *et al.* (15). This test, as adapted to individual mouse hemidiaphragms, has the advantage of not requiring any pipetting of individual samples, while all the reagents are added directly, some by automatic pipettes, to the colorimeter tubes in which the tissues are initially placed. However, the most important advantage of glycogen synthesis over glucose uptake as an indicator for insulin activity is that the percentage error in the measurement of glycogen increase is much less than that of glucose decrease. Other factors which increased the sensitivity of the test were the use of small (<20 g) rather than large (>30 g) mice, and the choice of an incubation time of 90 minutes which was long enough to allow maximum divergence in the glycogen levels of the insulin-treated tissues and the controls.

Antiserum was employed primarily for the purpose of increasing the precision of the test by increasing the slope of the dose-response curve. In fact, by this device it was possible to increase the slope 30-fold over that of the test without antiserum. The high precision of the new method is reflected in its low λ -value of 0.015, which is substantially lower than that reported for any other insulin assay procedure. Thus it is to be compared with the value 0.4 for the mouse convulsion test (18), 0.15 for the rabbit blood sugar assay (19), 0.59 for the mouse hemidiaphragm test using glucose uptake (5), and 0.45 reported here for the mouse hemidiaphragm test using glycogen synthesis.

With certain toxin-antitoxin systems (diphtheria and tetanus), the slope of the dose-response curve for toxin can be markedly increased if a constant amount of antitoxin is added to dilutions of toxin before the mixtures are injected into the test animals. Furthermore, the greater the antitoxin concentration in terms of toxin neutralized, the steeper is the slope. These principles apply also to the system insulin-anti-insulin. At low levels of anti-insulin (2 milliunits/ml) the dose-response slope is less steep than at higher levels. It was found, however, that there was no advantage in increasing the anti-insulin concentration beyond 10-20 milliunits/ml. A likely explanation for the slope having an upper limit is that the reaction of insulin with anti-insulin is incomplete. Thus, when both reactants are present in equivalent amounts and at high levels, the small proportion of insulin which is unneutralized is sufficient to stimulate the tissues. It is probable, therefore, that individual antisera will give the best slope with certain concentration ranges depending on the dissociation constant of the insulin-anti-insulin complex. A few specimens of anti-ox-insulin induced in the horse gave distinctly lower slopes than anti-ox-insulin induced in the guinea pig and this is attributed to the weaker binding between the horse antibody and insulin. Another difference between the antisera from the two species is that the guinea pig serum causes diabetes when administered to mice (8), whereas the horse serum does not (9).

The precision of an assay method is usually assessed from the internal

evidence of slope and standard deviation. An alternative and somewhat more laborious, although more realistic, method is to use the assay to titrate coded samples whose true potency is known to an independent referee, and then to compare the true potencies with the assay estimates. To the authors' knowledge the only insulin assay method with which this has heretofore been done is the rabbit blood sugar assay (20). With the present method, a series of 18 unknowns was titrated and it was found that the standard deviation of the estimates from the true values was 7%. This is a relatively small deviation in view of the number of mice used, which was only 230 for the whole experiment, or an average of 13 mice per sample including those used on the standard. The assays in this particular experiment were not run with maximum precision since they were not taken past the stage of interpolating the unknown responses onto the standard curves. To obtain better data, the next stage would have been to run a two- or three-dose assay at identical levels on standard and unknown, for this has been shown to give a standard deviation of 2 to 3% using six mice on standard and six on unknown.

To be acceptable, an assay method in addition to having good precision should also be specific for the substance being measured. The present method relies on two features for its specificity, namely, anti-insulin serum as a specific neutralizing reagent, and the diaphragm test as a biological indicator for unneutralized insulin. These two features make the assay highly specific for those mammalian insulins tested, as shown by the close agreement between the new method and the mouse convulsion test when insulins in different states of purity and from different species were titrated. The hemidiaphragm test with antiserum is not satisfactory, however, if the insulin has a different immunological specificity from ox insulin. This was shown with two samples of fish insulin where the assay gave false high values. Similar data have been obtained by the mouse convulsion test (21). Presumably this difficulty could be overcome if suitable antisera were available. It should be mentioned that with insulins of unknown immunological specificity, the hemidiaphragm test *without* antiserum, can be used to obtain a rough estimate of hormonal potency.

Emphasis in this investigation has been placed on the use of a biological indicator to follow the course of the insulin-anti-insulin titration. The reason is that commercial crystalline insulin is not a pure substance, but contains impurities which are highly antigenic. Thus certain anti-insulin sera may contain antibodies directed primarily against an impurity (22, 23). Therefore where the purpose is to assay both crude and purified insulins, it is desirable to use a specific biological system such as the diaphragm test, which detects hormone activity. It may be mentioned that Berson and Yalow, using a tracer method (7), found definite immunological differences between different mammalian insulins, for example between ox and pig, whereas in this investigation no such differences have been observed. This latter finding is in agreement with mouse convulsion data (8, 9, 10).

It is not suggested that this test renders the standard rabbit or mouse tests

obsolete, nor that it replaces other in vitro tests for certain purposes. It is suggested, however, that real application will be found in laboratories where it is desired to assay insulin to a high level of precision by a biological method but without an extensive outlay in animals and facilities. A further advantage is that only small quantities of insulin, in the region of 0.1 unit, are required, which is particularly useful if the material is scarce.

Acknowledgments

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STUDIES ON THE IN VITRO ANTITUMOR ACTIVITY OF FATTY ACIDS

III. SATURATED MONOCARBOXYLIC ACIDS¹

SUSAN TOLNAI AND JOSEPH F. MORGAN

Abstract

Previous studies on the in vitro antitumor activity of 10-hydroxy-2-decenoic acid from royal jelly and of saturated dibasic fatty acids have been extended to a series of saturated monocarboxylic acids ranging from C₃ to C₁₈. A widespread ability to inhibit the development of the ascites forms of the 6C3HED lymphosarcoma, Ehrlich carcinoma, and TA₃ mammary carcinoma has been found under acid pH conditions. In general, the antitumor activity of the saturated monocarboxylic acids was most pronounced in compounds with a carbon chain length of C₃ to C₁₄. One member of this series, capric (decanoic) acid, was found to possess marked antitumor activity at physiological pH.

Introduction

Previous studies from this laboratory have shown (1, 2) that royal jelly, when mixed with tumor cells prior to inoculation of mice, completely suppressed the formation of three lines of ascites tumors and the development of transplantable AKR leukemia. Fractionation studies (2) established that the antitumor activity resided mostly, if not entirely, in 10-hydroxy-2-decenoic acid, the main fatty acid component of royal jelly. Under strictly defined conditions, and at pH values below 4.5, tumor-inhibiting activity was demonstrated with saturated dicarboxylic acids in the series of chain length from C₃ to C₁₀ (3). In an effort to elucidate the possible relationship between the chemical structure of fatty acids and their antitumor activity, these investigations were extended to other types of fatty acids. The present communication reports the results obtained when monocarboxylic acids of chain length from C₃ to C₁₈ were tested for antitumor activity against three lines of ascites tumors in mice.

Materials and Methods

In vitro antitumor studies were carried out with three lines of ascites tumors: the 6C3HED lymphosarcoma, the Ehrlich ascites carcinoma, and the TA₃ mammary carcinoma. The 6C3HED lymphosarcoma was carried by weekly intraperitoneal passage in C3H mice. The other two ascites tumors were also carried by weekly serial passages, but in the non-specific Connaught mouse strain (4). All antitumor experiments were carried out in this non-specific mouse strain, using male mice, 20 to 22 g in weight. The C3H mice used in these experiments were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Connaught mice from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

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Contribution from the Biochemical Research Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

The ascitic material was harvested by intraperitoneal puncture 6 to 8 days after inoculation, mixed with two volumes of deionized water to lyse any erythrocytes present and the tumor cells collected by centrifugation at 1500 r.p.m. for 3 minutes. The packed cells were resuspended in sufficient Hanks' balanced salt solution (5) to yield a suspension containing 10 to 15 million cells per milliliter, as determined by direct cell count in a Spencer-Neubauer hemocytometer.

The monocarboxylic acids to be tested were incorporated in graded amounts in Hanks' solution, the washed cell suspension added, and the pH of the reaction mixture adjusted to the desired level with 0.1 *N* HCl or 0.1 *N* NaOH, using a Beckman pH meter. Complete details of these methods have been published previously (2).

When fatty acids containing 10 or more carbon atoms were to be tested, it was found necessary to use Tween 80 (obtained from the Atlas Powder Co., Wilmington, Delaware) in a 2% weight per volume concentration as an emulsifying agent. The Tween 80 was added to the Hanks' solution diluent used in preparing cell suspensions for the inoculation of both test and control mice and at this concentration was found to have no effect upon the survival time.

Groups of 10 mice were injected intraperitoneally with 0.5 ml per mouse of the reaction mixtures as rapidly as possible, and, at the same time, groups of control mice received the same number of tumor cells without admixture of the fatty acids. It was found that any deaths caused specifically by tumors occurred within the first 60 days following inoculation. Accordingly, all test mice were kept under daily observation for an arbitrary period of 90 days, at which time the experimental results were recorded. However, weekly observation of the mice was continued for a total period of 1 year, and at the end of this time all surviving animals were autopsied and examined for possible tumor growth.

The monocarboxylic acids used in these experiments were of the highest quality available commercially, and were used without further purification.

Results

In Vitro Antitumor Activity of Monocarboxylic Acids

In Table I are summarized the results of experiments carried out with Ehrlich ascites cells mixed with varying amounts of monocarboxylic acids ranging in chain length from C₃ to C₁₈. In all cases, the pH of the reaction mixtures was kept below 4.5. With the exception of butyric and valeric acids, all the fatty acids in this series caused complete protection against the inoculated tumor cells. Similar results were obtained when 6C3HED or TA₃ ascites tumor cells were used, but some minor differences in the responsiveness of the individual tumor types were observed.

Relationship between Concentration and Activity of Monocarboxylic Acids

In order to determine the relationship between concentration and activity of the monocarboxylic acids, each tumor cell type was tested over a wide range of

TABLE I
Effect of monocarboxylic acids when mixed with Ehrlich ascites carcinoma cells at pH 4.5 prior to inoculation of mice*

Fatty acid	Concentration (mg per ml)	Average survival (days)		Survivors (90 days)
		Test	Control	
Propionic (C ₃)	1.6	27†	11	2/10
	3.2	∞‡	12	9/9
Butyric (C ₄)	3.2	54	12	7/10
Valeric (C ₅)	3.2	28	10	8/10
Caproic (C ₆)	1.6	39	14	9/10
	5.0	∞	14	10/10
Caprylic (C ₈)	1.6	42	13	8/10
	2.5	∞	13	10/10
Capric (decanoic) (C ₁₀)	1.0	20	13	9/10
	2.0	∞	13	10/10
Lauric (C ₁₂)	1.0	22	15	8/10
	2.0	∞	15	10/10
Myristic (C ₁₄)	0.5	9	11	0/10
	1.0	∞	17	10/10
Palmitic (C ₁₆)	0.5	8	11	0/10
	1.0	∞	17	10/10
Stearic (C ₁₈)	1.0	27	17	0/10
	2.0	∞	15	10/10

*Each mouse received 5 to 8 million tumor cells either alone (control) or mixed with monocarboxylic acid (test).

†Calculated from the death times of the mice which failed to survive.

‡Represents survival beyond the 90-day test period.

fatty acid concentrations under acid pH conditions. Representative data are summarized in Table II. The amount of fatty acid required for complete suppression of tumor growth in most cases was only slightly higher than the partially protective or ineffective doses. However, the range between effective and ineffective concentrations was generally wider than that found with most dicarboxylic acids tested previously (3).

Variations in Tumor Susceptibility

Variations in susceptibility to the monocarboxylic acids were found among the three tumors, as summarized in Table III. Only two of the fatty acids tested, capric and myristic acids, protected the inoculated mice against all three ascites tumors at the same concentration. In general, the 6C3HED lymphosarcoma proved to be the most sensitive of the three tumors used and produced results similar to those obtained previously with the dicarboxylic acid series (3).

The minimal effective concentration, i.e., the smallest amount of fatty acid which gave total protection in all mice inoculated, could not be determined exactly with palmitic and stearic acids against the 6C3HED lymphosarcoma and the TA₃ mammary carcinoma. In these cases, the highest levels of the fatty acids tested (2.0 mg per ml) gave only partial protection, but since emulsi-

TABLE II
Effects of varying concentrations of representative monocarboxylic acids
when mixed with tumor cells prior to inoculation of mice*

Tumor cell	Monocarboxylic acid	Concentration (mg per ml)	Average survival (days)		Survivors (90 days)
			Test	Control	
6C3HED	Caproic	0.4	12	11	0/10
		0.8	25†	11	6/10
		1.6	∞‡	14	10/10
Ehrlich	Caprylic	0.8	15	13	0/10
		1.6	42	13	8/10
		2.5	∞‡	13	10/10
TA ₃	Capric (decanoic)	0.5	17	14	0/10
		1.0	28	14	7/10
		2.0	∞‡	15	10/10

*All experiments were carried out at pH 4.5. Each mouse received 5 to 8 million tumor cells alone (control) or mixed with monocarboxylic acid (test).

†Calculated from the death times of the mice which failed to survive for 90 days.

‡Represents survival beyond the 90-day test period.

TABLE III
Difference in susceptibility of ascites tumors to
monocarboxylic acids*

Monocarboxylic acid	Minimal effective concentration (mM)		
	6C3HED	Ehrlich	TA ₃
Propionic (C ₃)	21.6	43.2	43.2†
Butyric (C ₄)	18.1	36.3†	36.3†
Valeric (C ₅)	31.3	31.3†	31.3†
Caproic (C ₆)	13.7	43.1	43.1
Caprylic (C ₈)	5.5	17.3	5.5
Capric (C ₁₀)	11.6	11.6	11.6
Lauric (C ₁₂)	15.0	10.0	5.0
Myristic (C ₁₄)	4.3	4.3	4.3
Palmitic (C ₁₆)	7.8†	3.9	7.8†
Stearic (C ₁₈)	7.0†	7.0	7.0†

*All experiments were carried out at pH 4.5. Each mouse received 5 to 8 million tumor cells.

†Represents partial protection.

fication proved difficult to achieve at higher levels, experiments above the concentration indicated were not carried out. In tests with propionic, butyric, and valeric acids another type of difficulty was encountered. These three compounds proved to be readily soluble at all levels studied, but the relatively high concentrations required for activity against the Ehrlich and TA₃ ascites cells made it impossible to maintain the pH of the reaction mixtures above 3.5 without major alterations in the buffer system employed. Since pH values below 3.5 have been shown to be deleterious to the tumor cells (3), further experiments with these three fatty acids were not carried out.

Effect of pH on Activity of Monocarboxylic Acids

As noted in previous communications (1, 2, 3), the in vitro antitumor activity of the fatty acids tested was limited to a narrow range of pH values:

only mixtures at pH 4.5 or below proved to be effective against the ascites tumor cells. Each of the present series of monocarboxylic acids was tested over a wide range of pH values with the three experimental tumors. The same general limitation was found: antitumor activity could be demonstrated only at pH values below 4.5. However, one important exception to this general limitation was observed. Capric (decanoic) acid, alone of the monocarboxylic acids studied, was effective in suppressing the development of all three ascites tumors over a wide range of pH values. The results obtained with this fatty acid are summarized in Table IV. It is evident that decanoic acid, at concentrations comparable to that required for antitumor activity at acidic pH (Table III), is capable of suppressing the development of all three types of ascites tumors at the physiological pH of 6.8.

TABLE IV
Effect of capric (decanoic) acid when mixed with ascites tumor cells at pH 6.8 prior to inoculation of mice*

Tumor cells	Concentration (mg per ml)	Average survival (days)		Survivors (90 days)
		Test	Control	
6C3HED	2.0	22†	13	3/10
	4.0	∞‡	18	10/10
Ehrlich	2.0	11	17	0/10
	4.0	∞‡	11	10/10
TA ₃	1.0	14	14	0/10
	2.0	∞‡	15	10/10

*All determinations were carried out at pH 6.8. Each mouse received 5 to 8 million tumor cells either alone (control) or mixed with capric acid (test).

†Calculated from the death times of the mice which failed to survive for 90 days.

‡Represents survival beyond the 90-day test period.

Discussion

In previous investigations on the antitumor activity of fatty acids (1, 2, 3), two major drawbacks to therapeutic application were encountered. First, antitumor activity could only be demonstrated at pH values below 4.5 and, secondly, it was found necessary to mix the tumor cells with the fatty acids *before* inoculation into mice in order to achieve tumor suppression. The experiments reported in the present communication were devised to furnish additional data on the relationship between chemical structure and antitumor activity of fatty acids, and to continue the systematic search for fatty acids active at physiological pH. In the monocarboxylic acid series of chain length from C₃ to C₁₈, one such compound has been found, decanoic acid. This observation is deemed to be of considerable significance since the component of royal jelly exhibiting essentially all the antitumor activity of this material has been identified as 10-hydroxy-2-decenoic acid (1, 2).

In previous studies with the dicarboxylic acids (3), it was found that the antitumor activity increased progressively with increasing chain length of the fatty acids. In general, similar observations were recorded in the present study

with the saturated monocarboxylic acids. The greatest activity was found with compounds in the carbon chain length of C_8 to C_{14} , and tended to be less marked in compounds with carbon numbers above or below this range. Strict interpretation of this general observation is rendered difficult, however, by the marked variability of the three experimental tumors in their response to the individual fatty acids. The minimal effective concentrations of the monocarboxylic acids were found to be approximately equal to those of the dicarboxylic acids established previously (3).

The phenomenon of pH dependency in the antifungal activity of fatty acids is well known. Chattaway, Thompson, and Barlow (6) showed that the fungicidal activity of saturated fatty acids of chain length from C_2 to C_{14} increased with decreasing pH. Prince (7) concluded that the antifungal activity of undecylenic acid depended upon the undissociated molecule, since the fatty acid and its calcium salt showed equal activity between pH 4.5 and 5.0, but above this value the effective concentration was much higher in the case of the calcium salt. If the antitumor activity of fatty acids is at least partly dependent upon acidic pH, i.e., upon the undissociated molecule, then the members of the monocarboxylic acid series with higher chain lengths should show antitumor activity at higher pH values than those with less carbon atoms. Evidence to this effect was found previously among the dicarboxylic acids (3) and, in the present study, among the monocarboxylic acids. Thus, while propionic acid did not confer any protection above pH 3.9, lauric, myristic, and stearic acids gave positive results at pH 4.5. This finding is in agreement with the results of Hoffman, Schweitzer, and Dalby (8), who reported that while formic and acetic acids were fungistatic only at pH 2.0 to 3.0, fatty acids with three to six carbon atoms displayed activity at up to pH 5.0, and enanthic, caprylic, and pelargonic acids (C_7 to C_9) were fungistatic even at neutrality.

The present experiments do not explain the mechanism of the *in vitro* antitumor activity of fatty acids. It seems probable that the surface active properties of these compounds must play a major role in this activity. In addition, there is increasing evidence that activity is dependent, at least in part, upon the undissociated form of the molecule. Both these observations are of possible significance in view of the importance of dissociation properties and lipid solubility in the transport of drugs across cellular and intracellular barriers (9). Other factors which may have a bearing on the observed phenomena are the rapid oxidation of certain long-chain fatty acids by ascites cells (10, 11, 12) and the effect of fatty acids upon oxidative phosphorylation (11, 13).

The major finding of the present study has been the observation that capric (decanoic) acid, alone among the mono- and di-carboxylic acids tested, is effective in inhibiting tumor growth *in vitro* at the physiological pH value of 6.8. The minimal effective concentration of decanoic acid at pH 6.8 was found to be relatively low (2.0 mg per ml for TA₃ cells, and 4.0 mg per ml for 6C3HED and Ehrlich cells), and this would appear to indicate considerable tumor-suppressing ability. The discovery of a fatty acid with *in vitro* anti-

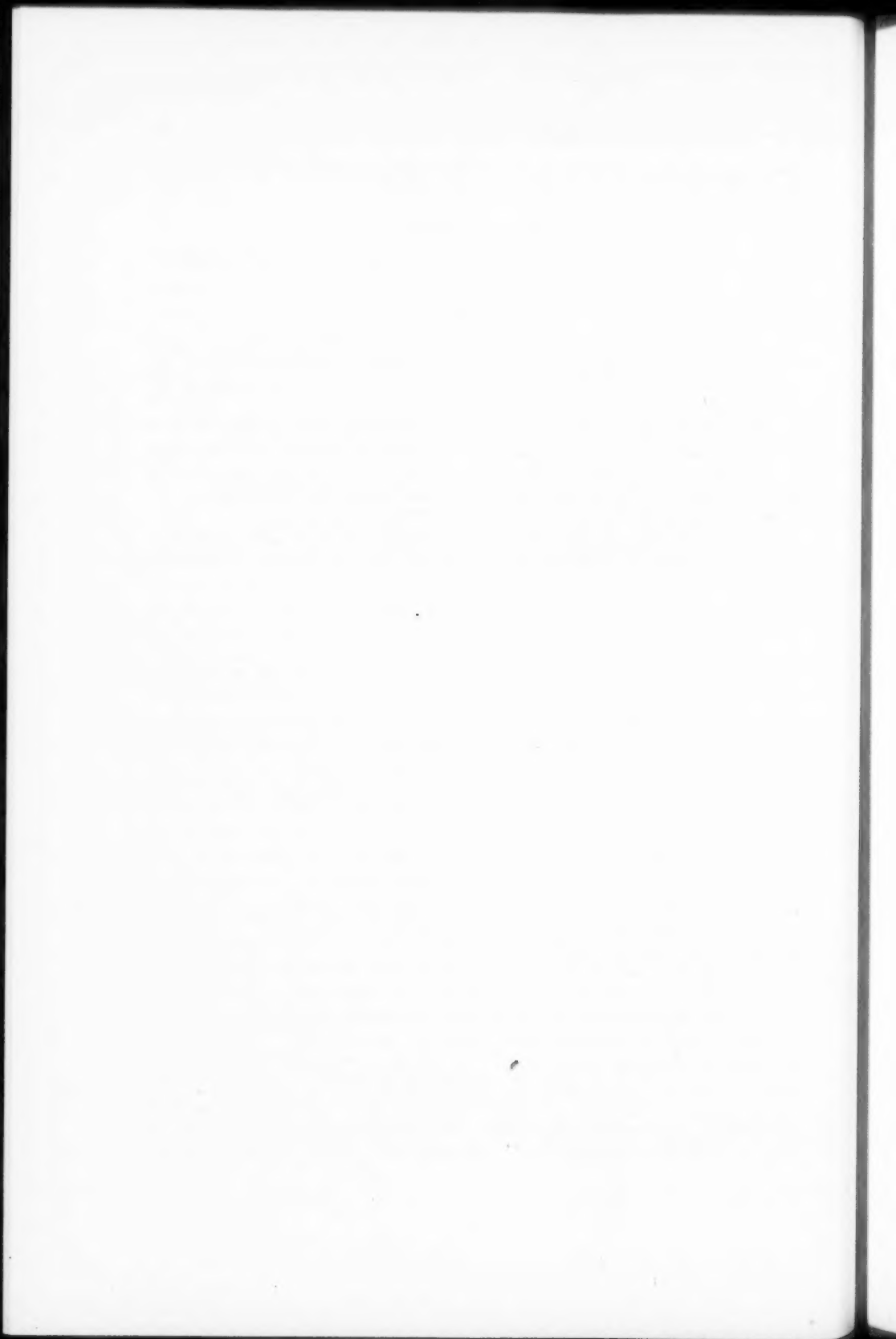
tumor activity demonstrable at physiological pH removes a serious drawback to possible therapeutic application of these studies.

Acknowledgment

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THE ISOLATION OF DINUCLEOTIDES FROM ALKALI HYDROLYZATES OF RIBONUCLEATES¹

B. G. LANE² AND FRANK WORTHINGTON ALLEN

Abstract

Improved methods for isolating the alkali-labile dinucleotides present in the alkali hydrolyzates of ribonucleates are presented.

Introduction

The hydrolysis of ribonucleates by alkali has been shown to result in the intermediate formation of alkali-labile dinucleotides (1).^{*} It was learned that the eight dinucleotides generally assumed to be resistant to hydrolysis by pancreatic ribonuclease (ApAp, ApGp, ApCp, ApUp, GpGp, GpAp, GpCp, and GpUp) accounted for the major portion of the alkali-labile dinucleotides found in the neutralized hydrolyzates. An extensive study by Dimroth and Witzel of the hydrolysis of ribonucleates catalyzed by bismuth hydroxide also indicated a clear correlation of the specificity of this type of chemical hydrolysis with that catalyzed by pancreatic ribonuclease (2). It is the intention of the present report to describe the details of a much simplified procedure for isolating the eight dinucleotides mentioned above and to extend some of the observations of an earlier report (1).

Materials and Methods

Materials

Ribonucleates were obtained from calf liver by the method of Pain and Butler (3). Commercial ribonucleates were purchased from Schwarz Laboratories. Pancreatic ribonuclease was purchased as a crystalline, protease-free preparation from Worthington Biochemicals. DEAE-cellulose was purchased as Selectacel from Brown Co.

Methods

(i) Hydrolysis of Ribonucleates

Ten grams of ribonucleates were hydrolyzed in 100 ml of 1.0 *M* potassium hydroxide for 2 hours at 25°. The hydrolyzate was adjusted to pH 7 with concentrated perchloric acid and the potassium perchlorate was removed by centrifugation. The sediment was re-extracted with 100 ml of distilled water and centrifuged, and the supernatant solution was pooled with the supernatant

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Contribution from the Department of Biochemistry, University of California School of Medicine, San Francisco, California, U.S.A.

²Present address: The Rockefeller Institute, New York 21, New York, U.S.A.

^{*}See reference 1 for abbreviations which have been used. Pu and Py are used as symbols for purine nucleoside and pyrimidine nucleoside, respectively. Internucleotide phosphodiester bridges are presumed to be 3'-5' linkages unless otherwise indicated.

solution from the first centrifugation. The combined supernatant solutions were frozen and thawed, and the potassium perchlorate which precipitated was removed by centrifugation.

(ii) *Separation of Dinucleotides from the Other Products of Hydrolysis of the Ribonucleates*

The neutralized hydrolyzate was diluted to 1 liter and charged under gravity onto a 33 cm \times 4.5 cm column of DEAE-cellulose (in the formate form) which had been pre-equilibrated with 0.025 *M* ammonium formate, pH 5.1. Details of the preparation and operation of DEAE-cellulose columns have been given previously (1). The charging solution passed through the column at a flow rate of 325 ml per hour and then elution, under pressure, was continued at a rate of 1300 ml per hour with stepwise increases in the salt concentration of the influent. Mononucleotides were eluted with 0.075 *M* ammonium formate, pH 5.1, and the bulk of the dinucleotides was eluted with 0.150 *M* ammonium formate, pH 5.1.

Desalting and concentrating of the dinucleotides in all chromatographic fractions were effected by published procedures (1) using Norit A charcoal.

(iii) *Separation of Individual Dinucleotides*

The chromatographic separations of the dinucleotides were effected using DEAE-cellulose columns under conditions similar to those described previously (1). The mixtures of dinucleotides were adsorbed from aqueous solutions of 0.025 *M* ammonium formate, pH 5.1, onto columns which had been pre-equilibrated with 0.025 *M* ammonium formate, pH 5.1.

(iv) *Identification of Dinucleotides*

The dinucleotides were identified by an assortment of degradative procedures (1) and also by spectral ratios at pH values of 2 and 12, which were reported for the corresponding dinucleoside phosphates by Dimroth and Witzel (2).

(v) *Determination of the Quantity of Dinucleotides Susceptible to Pancreatic Ribonuclease in the Chromatographic Fractions*

Thirty milliliters of 0.1 *M* TRIS (Tris(hydroxymethyl)aminomethane) buffer, pH 7.2, containing 200 mg of a lyophilized preparation of the dinucleotides and 4.5 mg of pancreatic ribonuclease were incubated at 37° for 3.5 hours. The digest was diluted to 200 ml and applied to a 20 cm \times 2.5 cm column of DEAE-cellulose pre-equilibrated with 0.025 *M* sodium acetate, pH 5.4. Mononucleotides were eluted with 0.075 *M* sodium acetate, pH 5.4, and dinucleotides were eluted with 0.150 *M* sodium acetate, pH 5.4. An approximate measure of the quantity of dinucleotides susceptible to pancreatic ribonuclease was provided by the quantity of mononucleotides obtained by the procedure outlined above.

Results

The Initial Separation of Dinucleotides from Other Hydrolysis Products

The initial fractionation of the neutralized alkali hydrolyzate which is indicated in Fig. 1 effected a separation of dinucleotides from mononucleotides and

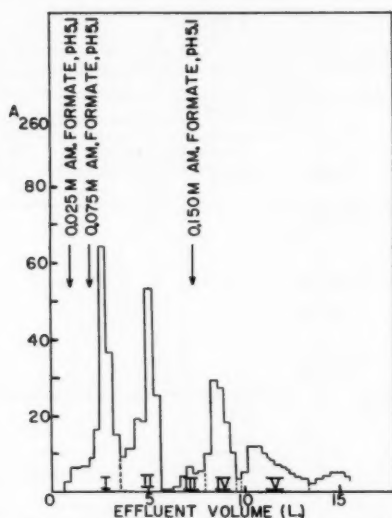


FIG. 1. Chromatographic separation of dinucleotides from the mononucleotides and higher oligonucleotides present in a neutralized alkali hydrolyzate of 10 g of commercial ribonucleates (see text for details).

Fraction I: Up, Cp; fraction II: Ap, Gp; fraction III: CpCp, CpUp, UpUp, UpCp; fraction IV: ApCp, ApUp, GpCp, GpUp, CpAp, UpAp, CpGp, UpGp; fraction V: ApAp, ApGp, GpGp, GpAp.

higher oligonucleotides. The mononucleotides were eluted in fractions I (Up and Cp) and II (Ap and Gp). The dinucleotides were eluted in fractions III (CpCp, CpUp, UpUp, and UpCp), IV (ApCp, ApUp, GpCp, GpUp, CpAp, UpAp, CpGp, and UpGp), and V (ApAp, ApGp, GpGp, and GpAp). Thus, fraction III contained PypPyp dinucleotides, fraction IV contained PupPyp and PypPup dinucleotides, and fraction V contained PupPup dinucleotides.

The Quantities of the Dinucleotides in Terms of their Susceptibility to Pancreatic Ribonuclease

The dinucleotides ApCp, ApUp, GpCp, and GpUp comprised about 80% of the dinucleotides of fraction IV as judged by the percentage conversion of the compounds of the fraction to mononucleotides when treated with pancreatic ribonuclease. The relative amounts of the dinucleotides in the alkali hydrolyzates of ribonucleates in terms of their susceptibility to pancreatic ribonuclease are given in Table I.

Chromatographic Separation of Individual Dinucleotides

The re-chromatography of the dinucleotide fraction (fractions III, IV, and V pooled), using DEAE-cellulose in the acetate form, eluted with 0.100 M sodium acetate, pH 4.6, is illustrated in Fig. 2. As would be anticipated, the elution pattern was generally similar to that seen in Fig. 1. The PypPyp dinucleotides were eluted together in a single small peak and were immediately followed by

TABLE I
Classification of the dinucleotides in alkali hydrolyzates of ribonucleates
in terms of their susceptibility to pancreatic ribonuclease

Classification	Type	Relative amounts of each type in:	
		2-hour hydrolyzate of commercial ribonucleates (%)	2.5-hour hydrolyzate of calf liver ribonucleates (%)
Susceptible to hydrolysis by pancreatic RNase	PypPyp	10	7
	PypPup	10	11
Not susceptible to hydrolysis by pancreatic RNase	PupPup	41	46
	PupPyp	39	36

NOTE: The dinucleotide fractions of the commercial and calf liver hydrolyzates accounted for 25% and 27% of the total ribonucleates, respectively.

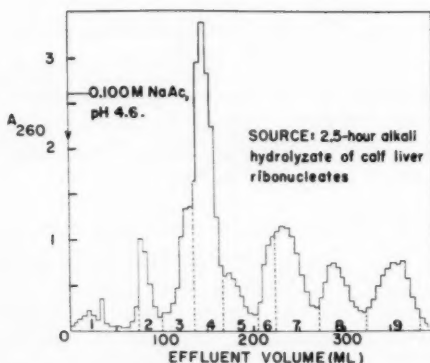


FIG. 2. Re-chromatography of about 20 μ moles of the dinucleotides (fractions III, IV, and V pooled) from a neutralized 2.5-hour alkali hydrolyzate of calf liver ribonucleates using a 30 cm \times 1.0 cm DEAE-cellulose column with a flow rate of 5 ml per hour.

(1) Mononucleotides; (2) CpCp, CpUp, UpUp, UpCp; (3, 4, 5) ApCp, ApUp, GpCp, GpUp, CpAp, CpGp, UpAp, UpGp; (6) ApAp; (7) ApAp, GpAp; (8) ApGp; (9) GpGp.

a large peak containing the PupPyp and PypPup dinucleotides. The PupPup dinucleotides were partially resolved with ApAp and GpAp appearing in a single peak and were followed by a peak containing ApGp and another peak containing GpGp. The separation of ApAp from GpAp can be effected by chromatography at pH 2.0 as indicated in Fig. 3.

Thus, the PupPup dinucleotides can be separated by subjecting fraction V to chromatography on DEAE-cellulose at pH 2.0 which separates ApAp from the remaining PupPup dinucleotides and the latter compounds can be resolved by chromatography on DEAE-cellulose at pH 4.6.

The PupPyp dinucleotides (fraction IV treated with pancreatic ribonuclease

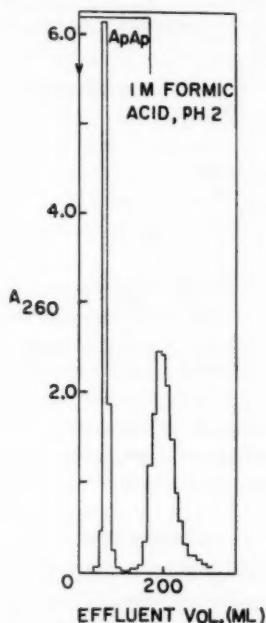


FIG. 3. Chromatographic separation of ApAp from about $20 \mu\text{moles}$ of the PupPup dinucleotides (fraction V) using a $50 \text{ cm} \times 1.2 \text{ cm}$ DEAE-cellulose column and a flow rate of 50 ml per hour.

and freed of the resulting mononucleotides) can be resolved by chromatography at pH 2.0 as indicated in Fig. 4.

Hydrolysis of Dinucleotides in Alkali

The hydrolyses of dinucleotides follow first-order kinetics for fixed concentration of alkali. The dinucleotides ApUp, GpCp, and GpUp have rate constants of about $0.4 \text{ (hour}^{-1}\text{)}$ for hydrolysis in 0.86 potassium hydroxide at 29° while the rate constants for ApAp and ApCp are about 0.2. Owing to the marked dependence of the rate constants on temperature (4) the absolute values have been quoted with only one-figure accuracy in the absence of accurate measurements of temperature. An investigation of the temperature dependence of the individual rate constants will be undertaken but in the meantime it seems advisable to indicate that the values quoted in an earlier paper (1) have relative significance only in the region of 25° since the experiments were conducted at constant temperature to ascertain relative rates of hydrolysis, no attempt having been made to measure the precise temperature with a suitably calibrated instrument.

It seems from the data which have been collected that the substitution of an amino group for a keto group at position 6 of the pyrimidine ring markedly

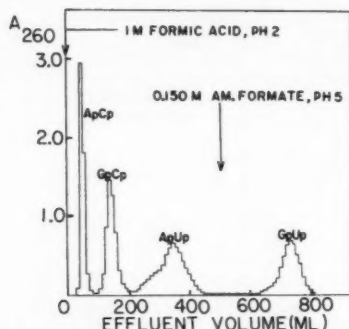


FIG. 4. Chromatographic separation of about 20 μ moles of the PupPyp dinucleotides using a 50 cm \times 1.2 cm DEAE-cellulose column and a flow rate of 50 ml per hour.

decreases the rate of hydrolysis of the internucleotide linkage judging by the facts that UpUp hydrolyzed more rapidly than CpCp (1) and that ApUp hydrolyzed much more rapidly than ApCp.

Discussion

Chromatographic Separation using DEAE-Cellulose

The group separation of mononucleotides, dinucleotides, and trinucleotides is most effective on the basis of charge differences at pH values of about 5 (5). Separations of oligonucleotides on Dowex-1 in the range of pH values between 1 and 3 (6, 7, 8, 1, 2) or on DEAE-cellulose at pH values between 8 and 9 (9) do not effect such group separation; the dinucleotide ApCp, for example, is eluted prior to the complete elution of mononucleotides at the acidic pH values used commonly for eluting from Dowex-1 (6). Sinsheimer used Dowex-1X2 and eluants at pH 5.5 to effect an almost complete separation of the dinucleotides from the mononucleotides and trinucleotides formed by the treatment of deoxyribonucleates with pancreatic deoxyribonuclease (10). The employment of DEAE-cellulose rather than Dowex-1 for anion-exchange fractionation at pH values about 5 (11, 1) has the advantage that there is a fairly discrete fractionation of the dinucleotides into three general types: (i) PypPyp; (ii) PupPyp, PypPup; and (iii) PupPup.

The Hydrolysis of Ribonucleates and their Derivatives in Alkali

The work of Brown and Todd (12, 13, 14) and of Markham and Smith (15, 16) established that the hydrolysis of ribonucleates effected by both pancreatic ribonuclease and by alkali proceeded via the intermediate formation of a cyclic phosphodiester bridge between the 2' and 3' positions of the ribonucleotides. At about the same time that the similarity between the mechanisms of hydrolysis by pancreatic ribonuclease and by alkali was being established, reports appeared in the literature which indicated a possible correlation between the specificities of these catalyses. Zittle had reported (17) that the ribo-

nuclease-resistant fraction of ribonucleates was hydrolyzed much more slowly by alkali than were the parent ribonucleates. Magasanik and Chargaff reported that during hydrolysis of ribonucleates by alkali, uridylic acid was liberated most rapidly and adenylic acid was liberated most slowly (18). A similar result was later reported by Montreuil and Boulanger (19).

Kinetic studies of the hydrolysis of ribonucleates in alkali by Cavalieri (20) and by Bacher and Kauzmann (4) suggested that all internucleotide linkages did not hydrolyze at the same rate and this result was subsequently supported by the data of Hakim (21).

The identification and kinetic studies of the alkali-labile dinucleotides present in alkali hydrolyzates of ribonucleates have extended these earlier observations and established a clear correlation between the specificities of pancreatic ribonuclease and hydroxyl-ion-catalyzed hydrolyses.

The individual dinucleotides isolated from the alkali hydrolyzates are isomeric mixtures bearing 2' and 3' phosphomonoester groups, and inasmuch as the hydrolyses of the isomeric mixtures have consistently followed first-order kinetics, it seems that the rates of hydrolysis of the isomers are similar. Lane and Butler reported (1) rate constants of 0.62 and 0.38 for two isomers of dicytidylate and it was proposed that these isomers differed only in the position of the monoester phosphate. It now seems more likely that one compound had a 2'-5' phosphodiester bridge (22, 23) and the other had a 3'-5' phosphodiester linkage. This interconversion of 2'-5' and 3'-5' linkages occurs in 0.3 *M* HCl, 40° (2), and it seems not unlikely that the treatment of the hydrolysis products of polycytidylate used previously (0.1 *M* HCl, 23°) may have effected a degree of interconversion. The separation of the two dicytidylate isomers by anion exchange chromatography is also consistent with the report by Dimroth and Witzel (2) and by Witzel (24) that a dinucleoside phosphate with a 2'-5' linkage is separated from the isomeric compound with a 3'-5' linkage on Dowex-1 resin.

It was found in the earlier kinetic studies (20, 4) of the hydrolysis of ribonucleates that more than one first-order rate constant was required to describe adequately the hydrolysis at low temperatures. It was considered that this effect reflected the different rates of hydrolysis of internucleotide linkages and (or) a variation of hydrolysis rate with the varying chain length of the polymers during the course of the hydrolysis. The studies of the hydrolysis of the dinucleotides have indicated that even at this considerably lower level of complexity, it is necessary to be cautious regarding the interpretation of results since any given dinucleotide X_pX_p could be present in four different isomeric forms: X_2pX_2p , X_2pX_3p , X_3pX_2p , and X_3pX_3p . The dinucleotides isolated from acid hydrolyzates of ribonucleates by Merrifield and Woolley (7) would, for example, be expected to be present in all four isomeric forms. It will also be important in any detailed kinetic studies to demonstrate that during preparation of dinucleotides from alkali hydrolyzates of ribonucleates that any acidic conditions employed during isolation do not effect a conversion of 3'-5' phosphodiester linkages to 2'-5' linkages. Furthermore, a possible contamination

of the alkali-labile dinucleotides by alkali-stable dinucleotides must be considered (25, 1).

Acknowledgments

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THE PREPARATION AND PROPERTIES OF GUANIDINATED OVALBUMIN¹

A. F. S. A. HABEED²

Abstract

A study of the guanidination of ovalbumin with 1-guanyl-3,5-dimethyl pyrazole nitrate showed that at pH 9.5 the reagent reacts specifically with the ϵ -amino groups of lysine. Guanidination did not produce configurational changes that could be detected by sedimentation, diffusion, or electrophoresis.

Introduction

The guanidination of β -lactoglobulin using 1-guanyl-3,5-dimethyl pyrazole nitrate (1, 2) required the blocking of sulphhydryl groups in order to prevent aggregation of the protein attributed to the formation of intermolecular disulphide bonds. Since ovalbumin contains five sulphhydryl groups (3) in a fairly rigid compact molecule its behavior on guanidination is of interest.

Steven and Tristram (4) found that in native ovalbumin only three ϵ -amino groups of lysine were accessible for reaction with fluorodinitrobenzene but that, during serial ethanol denaturation, the molecule uncoils and the groups become progressively available for reaction. The relative reactivity of a proportion of the free amino groups of ovalbumin and the importance of the remainder in maintaining the native structure has also been reported by Maurer *et al.* (5). Treatment of ovalbumin with phenylisocyanate (6) caused 70% reaction of the free amino groups but no physicochemical data were reported on the modified protein.

This paper reports on a study of the effects of guanidination on ovalbumin.

Materials and Methods

Materials

Ovalbumin (OA) (twice crystallized) and *N*-ethylmaleimide (NEMI) were obtained from Nutritional Biochemicals Corporation. 1-Guanyl-3,5-dimethyl pyrazole nitrate (GDMP) was synthesized as described by Bannard *et al.* (7).

Guanidination Procedure

Preliminary results showed that though gelling occurred in 0.5 *M* GDMP solution at pH 9.5 and 0°, no gelling took place in 0.2 *M* or 0.3 *M* GDMP solution. The following procedure was adopted: GDMP was dissolved in few milliliters of water in an ice bath, pH was adjusted as necessary with 1 *N*

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²National Research Council Postdoctorate Fellow, 1959-60. Present address: Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

sodium hydroxide, and water was added to make the solution 0.25 *M* with respect to GDMP. Ovalbumin was dissolved to 3% concentration and the pH was readjusted if necessary; after reaction for the desired time the solution was dialyzed against phosphate buffer, pH 7.5 and μ 0.1. The free amino groups as well as the concentration of the protein solution were determined.

Analytical Procedures

The percentage of unreacted free amino groups was determined by ninhydrin colorimetric analyses (8) at 570 *mμ*.

Protein concentrations were obtained from refractive index increment measurements with a Brice-Phoenix differential refractometer.

For amino acid analyses, protein was hydrolyzed for 24 hours with 6 *N* hydrochloric acid in a sealed evacuated tube at 105° C. After removal of the acid under vacuum over sodium hydroxide the amino acids were determined in an aliquot with a Beckman Spinco amino acid analyzer (9).

The tyrosine/tryptophan ratio was determined by the spectrophotometric method (10).

Cysteine was determined on OA and GuOA by adding 2 ml of NEMI solution (0.5 mg/ml) prepared in 0.2 *M* sodium dodecyl sulphate solution in 0.1 *M* phosphate buffer, pH 7.2, to 2 ml of 1% protein solution in 0.1 *M* phosphate buffer, pH 7.2. Solutions containing NEMI but no protein and one containing the protein but no NEMI were included for comparison. The sulphhydryl content was calculated from the decrease in NEMI added to the protein by measuring the optical density of the solutions at 310 *mμ* (11) after 1/2, 1, and 3 hours' reaction.

Physical Methods

Electrophoretic analyses were carried out in a Spinco Model H electrophoresis apparatus at 0.5% protein concentration in barbital buffer, pH 8.6, μ 0.1. Mobilities were calculated from the descending boundaries.

Sedimentation measurements were performed at various protein concentrations for OA and GuOA in a Spinco Model E ultracentrifuge in phosphate buffer, pH 7.5, μ 0.1, at 59,780 r.p.m. and 20° C.

The diffusion coefficient was determined in the ultracentrifuge at 12,590 and 8,225 r.p.m. on 1% protein solution in phosphate buffer, pH 7.5 and μ 0.1, at 20° C. A boundary was formed initially in the synthetic boundary cell and the spreading of this boundary studied (12). The diffusion coefficient was calculated by the height-area method (12) and corrected to water at 20° C. Because of the low speed of the ultracentrifuge, no correction was applied for sedimentation.

The frictional ratio f/f_0 was calculated from the formula (5)

$$\frac{f}{f_0} = 10^{-8} \left(\frac{1 - V\rho_w}{S_{20,w} D_{20,w}^2 V} \right)^{1/3}$$

where V = partial specific volume,

ρ_w = density of water at 20° C,

$S_{20,w}$ = sedimentation coefficient at infinite dilution,

$D_{20,w}$ = diffusion coefficient for 1% protein solution, corrected for water at 20°; this was used instead of the value at zero protein concentration since it has been found that the diffusion coefficient for OA is independent of protein concentration (13).

The molecular weight of GuOA was measured by the Archibald procedure (14, 15) at 12,590 r.p.m. Values for OA and GuOA were also obtained from the sedimentation and diffusion coefficients.

Results and Discussion

Effect of pH

The extent of guanidination as a function of pH after 4 and 7 days' reaction is plotted in Fig. 1, which shows that at pH 9.5 guanidination of OA is maximal and that further increase in pH does not significantly affect the reaction. Since

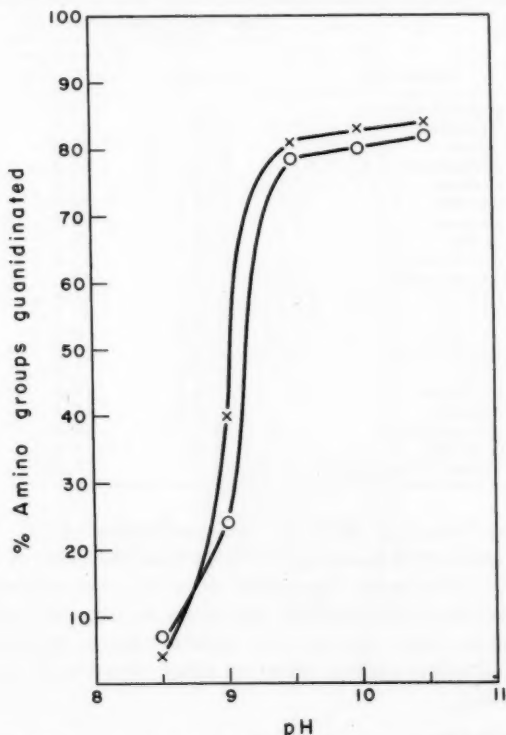


FIG. 1. Reaction of ovalbumin with GDMP. O—O reaction for 4 days; X—X reaction for 7 days.

only about 25% and 40% of the 20 amino groups of OA were guanidinated at pH 9 after 4 and 7 days respectively, possibly no more than eight groups are easily available for reaction and these may occupy positions on the surface of the molecule. Maurer *et al.* (5) found that 27–36% of the amino groups of OA are easily deaminated with little change in configuration while deamination of 33–56% led to an unfolding of the molecule, presumably because amino groups participating with free carboxylic groups in holding the protein in the native globular form are affected.

Extent of Guanidination

In Table I the amino acid analysis of GuOA is compared with values for OA, molecular weight 44,000. Only 18 out of 20 lysines were converted to homo-arginine. The incomplete reactivity may be due to steric hindrance. Resistant

TABLE I
The amino acid content of ovalbumin
and guanidinated ovalbumin

Amino acid	Mole amino acid/mole protein	
	Ovalbumin	Guanidinated ovalbumin
Aspartic acid	31.3	31.3
Threonine	13.2	14.0
Serine	26.5	29.8
Glutamic acid	48.6	47.5
Proline	14.3	14.3
Glycine	18.7	18.8
Alanine	36.2	34.9
Valine	31.9	29.1
Methionine	15.9	15.8
Alloisoleucine	0.6	0
Isoleucine	24.2	22.8
Leucine	32.2	32.0
Tyrosine	9.8	9.7
Phenylalanine	20.1	20.0
Lysine	20.7	1.8
Histidine	7.4	7.0
Arginine	15.0	15.3
Homoarginine	0	18.3
Cysteine	5.23	5.4
Tyrosine/tryptophan	2.7	2.66

lysines were also found in BSA and β -lactoglobulin (1, 2). The value for cysteine in OA and GuOA was slightly higher than the value of five sulphydryl groups reported (3); however, the results show that the sulphydryl groups of OA are unaffected by guanidination. Since the amino acid analysis of GuOA compares favorably with that of OA, GMP reacts specifically with the ϵ -amino groups of lysine without effect on other amino acids under the conditions of the reaction.

Effect on Configuration

Table II summarizes the physical measurements obtained on OA and GuOA.

TABLE II
Physical properties of ovalbumin and guanidinated ovalbumin

Protein	Electrophoretic mobility, $\text{cm}^2/\text{v}/\text{sec} \cdot 10^8$	$S_{20,w}$ at zero concentration	$D_{20,w}$ $\text{cm}^2/\text{sec} \cdot 10^7$	f/f_0	Molecular weight	
					From sedimentation and diffusion	From Archibald
OA	-6.22	3.45S	7.52 7.46*	1.2	39,500	—
GuOA	-6.76	3.35S	7.21	1.25	43,000	41,500

*Value obtained by operating the ultracentrifuge at 8225 r.p.m.

The sedimentation patterns showed a single sharp boundary indicative of a monodisperse system. Figure 2 shows the sedimentation coefficients of OA and GuOA as a function of concentration. The sedimentation coefficients extrapolated to zero concentration are 3.45 and 3.35 for OA and GuOA, respectively, and there is little dependence on concentration.

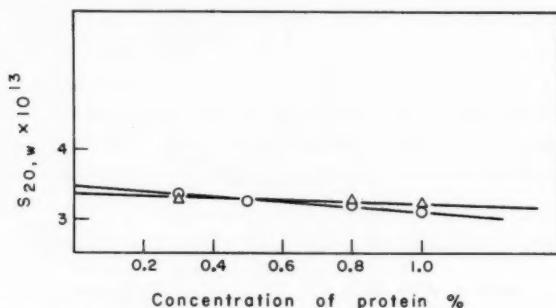


FIG. 2. Sedimentation coefficient as function of concentration. \circ — \circ ovalbumin; \triangle — \triangle guanidinated ovalbumin.

The electrophoretic mobility of GuOA is slightly more negative than the native protein, which may be due to binding of anions by the guanidino group. Similar behavior has been found with guanidinated chymotrypsinogen (16) and lactoglobulin (2). The magnitude of the change in the mobility of GuOA is less than that found with guanidinated lactoglobulin and may arise from configurational differences between the two proteins.

The results of sedimentation, diffusion measurements, and frictional ratio calculation fail to show any change in the shape of the molecule of GuOA. Since the molecular weights of OA and GuOA were similar, aggregation or degradation of the molecule did not occur upon guanidination. The replacement of the positively charged amino group by a positively charged guanidino group in OA results in little, if any, configurational changes as it avoids the change of charge of the protein molecule. Similar results were found with BSA (1, 2). In contrast, deamination of 33 to 56% of the amino groups resulted in the removal of the positively charged amino groups, which may have the function of participating with free carboxyl groups in holding the protein in the native globular form by hydrogen bond, and thus led to unfolding of the molecule. The deaminated derivative showed an increase in the negative optical rotation, a very marked decrease in the diffusion coefficient, and an increase in sedimentation coefficient (5), all indicative of unfolding of the molecule.

Denaturing Effect of GDMP

In addition to the guanidinating action of GDMP, it has a denaturing effect on proteins (similar to the action of guanidine and urea) which differs with the protein and also with the concentration of GDMP. This gelling occurs even at

the low pH of 8 where guanidination is at a minimum and is thus due to a denaturing effect on the protein and not due to the substitution of resistant lysines at high concentration of GDMP. Ovalbumin was guanidinated without blocking the sulphhydryl groups whereas lactoglobulin required the reaction of the sulphhydryl groups to prevent gelling (1, 2). A similar resistance to change in optical rotation of OA was noted in 7.3 *M* urea (17) and in 2.48 *M* guanidine (18) while with lactoglobulin there was an instantaneous change in rotation followed by a time-dependent change (19). Presumably because of the compact structure of OA, no unfolding of the molecule takes place in 0.3 *M* GDMP, but at 0.5 *M* the molecule uncoils and intermolecular disulphide bonds result in gelling. On the other hand, these changes take place in lactoglobulin even in 0.2 *M* GDMP solution unless the sulphhydryl groups were reacted beforehand with NEMI or iodoacetic acid (2).

Bovine serum albumin in 8 *M* urea (19) shows an instantaneous change in optical rotation and an increase in reduced viscosity (20). These changes are reversible on removal of urea. The lack of gelling of BSA in 0.5 *M* GDMP (1, 2) may depend on the inability to form intermolecular disulphide bonds due to low content of sulphhydryl which tends to decrease with time (21). If any unfolding of BSA had taken place in GDMP solution, the dialysis of the reaction mixture would result in its reversal and one would expect no configurational changes in GuBSA.

These differences, in response to the denaturation effect of GDMP and similar reagents, probably reflect differences in reactive sulphhydryl groups available for the formation of intermolecular disulphide bonds.

Acknowledgments

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THE EFFECT OF FORMALIN AND OF L-TARTARIC ACID ON SOME TISSUE ACID PHOSPHATASES¹

G. E. DELORY AND MERLE HETHERINGTON

Abstract

The inhibitory effect of 0.5% formalin and of 0.02 *M* L-tartaric acid has been studied on the acid phosphatase activity of a number of human tissue extracts. It was found that the sum of the formalin resistant and of the tartaric acid resistant enzyme activity closely approximated the activity of the uninhibited enzyme.

Introduction

Some years ago, we reported that the acid phosphatase activities of male and female urine were alike in their behavior towards the inhibitory substances formalin and L-tartaric acid (1).

Since then we have been studying the effects of these substances on human tissue extracts, which it is now considered of interest to report.

Methods

Extracts of fresh tissues obtained either by biopsy or at autopsy were prepared as follows:

The tissues were cut into small pieces, washed thoroughly with cold water to remove as much blood as possible, dried between filter papers, and homogenized with a volume of saline equal (in milliliters) to 20 times the tissue weight. These extracts were then treated with a few drops of toluene and chloroform, allowed to autolyze for 2 days, and filtered before use.

The acid phosphatase activities of these extracts were determined by the method previously described (2).

Results

The results shown in Table I indicate that the sum of the formalin resistant and of the tartaric acid resistant activities closely approximates to that of the total activity.

All determinations were carried out in duplicate and the means of the results were shown.

Discussion

The results suggest that all the tissues studied contain two acid phosphatases, one of which is formalin resistant and the other L-tartaric acid resistant, but

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Contribution from the Biochemistry Department, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba.

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TABLE I

Acid phosphatase activities of various origins before and after treatment
with 0.5% formalin and 0.02 M tartaric acid
(All values are expressed as mg P liberated per 100 ml of original solution)

Source	Total acid phosphatase A	Formalin resistant acid phosphatase B	Tartaric acid resistant phosphatase C	B + C
Liver	7.8 3.3	5.5 2.2	2.5 1.0	8.0 3.2
Dialyzed urine (males)	2.9 16.0	2.5 15.4	0.5 0.7	3.0 16.1
Dialyzed urine (females)	2.3 1.8	2.2 1.6	0.1 0.1	2.3 1.7
Kidney	6.0 2.3	4.0 1.5	2.0 0.8	6.0 2.3
Spleen	2.2 2.0	1.3 1.3	1.1 0.5	2.1 1.8
Adrenal	4.3 3.8	4.0 3.5	0.3 0	4.3 3.5
Washed red cells	10.4 5.9	0.1 0	9.9 5.9	10.0 5.9
Seminal fluid	7.1 42.6	6.8 39.4	0.3 1.7	7.1 41.1
Prostate	178.0 1540.0	172.0 1460.0	6.0 50.0	178.0 1510.0

further knowledge must await separation of the enzymes. To investigate this point further, experiments were carried out in which both tartaric acid and formalin were incorporated in the reaction mixture; but in no case was any residual activity detected.

Acknowledgment

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ACETATE METABOLISM IN EXPERIMENTAL KETOSIS OF GUINEA PIGS¹

FRANK SAUER

Abstract

Non-diabetic ketosis was produced experimentally in fasted pregnant guinea pigs. Total CO₂ output of ketotic animals was less than that of appropriate controls but there was no impairment in the conversion of acetate-1-C¹⁴ to C¹⁴O₂. Sterol synthesis increased in ketotic animals while fatty acid synthesis, particularly in carcass, showed the expected decrease. Ketosis was accompanied by an increase in plasma total fatty acids and in the fatty acid concentration of liver. The experimental findings support the hypothesis that ketosis is a manifestation of increased ketogenesis rather than impaired utilization of ketone bodies.

Introduction

The concentration of ketone bodies in blood increases during starvation, following the feeding of a high-fat diet, and in pathological states such as diabetes mellitus, bovine ketosis, and ovine pregnancy toxemia. The relationship of oxalacetate and its precursors to the accumulation of acetoacetate has been thoroughly investigated (1-3). The suggestion that oxalacetate depletion is not the primary cause of ketosis (4) finds support in the observations of Shaw and Tapley (5) and Kalnitsky and Tapley (6) that increased acetoacetate concentration in livers of starved or diabetic rats was not accompanied by a decrease in oxalacetate content. It has been postulated (7-9) that impaired conversion of acetoacetyl-CoA to fatty acids results in increased acetoacetate formation: Segal *et al.* (10), however, have presented evidence that diabetic ketosis is accompanied by increased activity in enzyme systems converting acetoacetyl-CoA to acetoacetate. Wieland *et al.* (11) recently have shown that acetoacetate-synthesizing enzymes, particularly β -hydroxy β -methylglutaryl-CoA (HMG-CoA) condensing enzyme, show increased activity in diabetic ketosis. It is therefore unlikely that ketosis can be related simply to an accumulation of acetoacetate precursors.

Ruminant ketosis, unlike diabetic ketosis, is characterized by hypoglycemia and is frequently associated with reduced feed intake.

The physiological changes that are characteristic of the fasting state in some respects resemble those of ketosis, and indeed it is possible to produce experimental ketosis by fasting late pregnant ewes (12, 13) and guinea pigs (14, 15). However, the relationship between ketosis and fasting is poorly understood. The large increase in concentration of blood ketone bodies and the high mortality that occur in experimental ketosis of pregnancy are not characteristic of the fasting state. Recent reports (11, 16) indicate that the activities of some

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Contribution No. 53, Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

hepatic enzymes may be different during fasting and diabetic ketosis. Wieland *et al.* (11) observed that the activity of HMG-CoA reductase and acetoacetate-synthesizing enzymes are greater in diabetic ketotic than in fasted animals. Although these findings may not be directly applicable to non-diabetic ketosis, nevertheless they indicate that ketotic and fasting states may be characterized by differences in enzyme activities.

In the present work acetate metabolism was studied in fasted and ketotic animals. Bergman and Sellers (14) noted that pregnant guinea pigs develop hypoglycemia and ketosis when fasted. This procedure was used to provide ketotic animals for these studies.

Materials and Methods

Adult guinea pigs weighing $864 \pm 29.3^*$ g were used for in vivo and in vitro experiments.

In the in vivo experiments (Tables I-VI) acetate-1- C^{14} (260×10^6 c.p.m.) was injected intraperitoneally. Immediately after acetate injection animals were placed in metabolism chambers for 3 hours for CO_2 collection. The animals were then killed, eviscerated, the skin removed, and liver and carcass saponified for sterol and fatty acid isolation by a modification (17) of the method of Van Bruggen *et al.* (18). The alkaline tissue digest was brought to known volume and suitable aliquots were taken for petroleum ether extraction. The specific activities of fatty acid, sterol, and barium carbonate fractions were corrected for dose differences in order to permit comparison of experiments.

Animals in the non-pregnant starved (N.P.S.) group were fasted 72 hours. Animals of the ketotic group were fasted 72 hours or less and were used for experiments if the concentration of acetoacetate in blood fell within an arbitrary range of 5 to 18 mg per 100 ml. Animals with more than 20 mg acetoacetate were generally not suitable because of their high mortality.

Blood was taken by cardiac puncture under light ether anaesthesia. Total plasma fatty acids (Table VI) were determined by microtitration by the method of Albrink (19). Blood acetoacetate concentration was determined by the Kalnitsky and Tapley modification (6) of the method of Walker (20).

The in vitro experiments (Table VII) were carried out with perfused liver homogenates incubated in the phosphate-sucrose buffer of Bucher (21) as described previously (17). Net synthesis of acetoacetate and C^{14} activity in the carboxyl carbon of acetoacetate were determined as before (17).

It was shown by Chou and Lipmann (22) that fatty acid - CoA derivatives react non-enzymatically in the presence of high concentrations of hydroxylamine to form the corresponding hydroxamic acid derivatives. Three milliliters of liver homogenate (final volume, 4.5 ml) was incubated with 7.5 μ moles DPN, 3 μ moles ATP, 50 μ moles acetate, and 2 mmoles hydroxylamine (final pH 7.4) for 90 minutes at 37° C in O_2 . Water-soluble hydroxamic acid derivatives were estimated by Kornberg and Pricer's modification (23) of the method

*Standard error of mean.

of Lipmann and Tuttle (24). Acetylhydroxamic acid prepared by the method of Lipmann and Tuttle (25) was used as standard.

Results

Data presented in Table I show that total CO_2 production was significantly decreased in ketotic animals but that the percentage of tracer incorporated into CO_2 did not differ significantly when compared with pregnant fed (P.F.)

TABLE I
Tracer incorporated into CO_2 and CO_2 produced during 3-hour period
following intraperitoneal injection of acetate-1- C^{14}

Group	mg AcAc/ 100 ml blood	CO_2 output mmoles/ hr/100 g	% incorporation
Ketotic (8)	$12.6 \pm 2.46^\dagger$	$1.28 \pm .069^\dagger$	$52.06 \pm 4.51^\dagger$
Pregnant fed (5)	<1	$2.11 \pm .087^{**}$	40.00 ± 4.54
Non-pregnant fed (5)	<1	$1.65 \pm .118^*$	48.55 ± 1.24
Non-pregnant starved (6)	1.8 ± 0.24	$1.67 \pm .051^{**}$	$64.75 \pm 0.81^*$

NOTE: Numbers in parentheses indicate number of animals used. Acetoacetate (AcAc).

Differs significantly from ketotic group where indicated:

* $P < .05$.

** $P < .001$.

† Standard error of the mean.

and non-pregnant fed (N.P.F.) animals. Cockburn and Van Bruggen (26) noted that the percentage of C^{14} from acetate-1- C^{14} incorporated into CO_2 increased with fasting. The present data, in agreement with this, show that the percentage of tracer incorporated into CO_2 by non-pregnant starved (N.P.S.) animals was significantly greater than in either non-pregnant fed ($P < .001$) or ketotic animals ($P < .05$). The specific activity of the expired CO_2 (Fig. 1) was lowest in the P.F. group, approximately the same for the ketotic and N.P.F. groups, and highest in the N.P.S. group. These data, in agreement with published reports (18, 27), indicate that while non-diabetic ketosis and starvation are characterized by decreased CO_2 production, acetate activation and oxidation proceed unimpaired. The suggestion has already been made (26) that these results could be accounted for by a decrease in availability of endogenous acetate (i.e. reduced 'acetate pool' size) and subsequently an increased utilization of added tracer acetate.

Data on the conversion of acetate-1- C^{14} into digitonide precipitable sterols of liver and carcass are presented in Tables II and III. The specific activity and percentage of tracer incorporated into liver sterols were increased in animals of the ketotic group. It is unlikely that these results are simply due to an increased specific activity of the 'acetate pool' because C^{14} incorporated into fatty acids (Tables IV and V) was not increased in the ketotic group and the specific activity of the fatty acids was lower than that of the N.P.F. and N.P.S. groups. The 72-hour fasted group did not show the decrease in sterol synthesis that has been reported for intact fasted rats (18) or liver homogenates prepared from young, fasted guinea pigs (17).

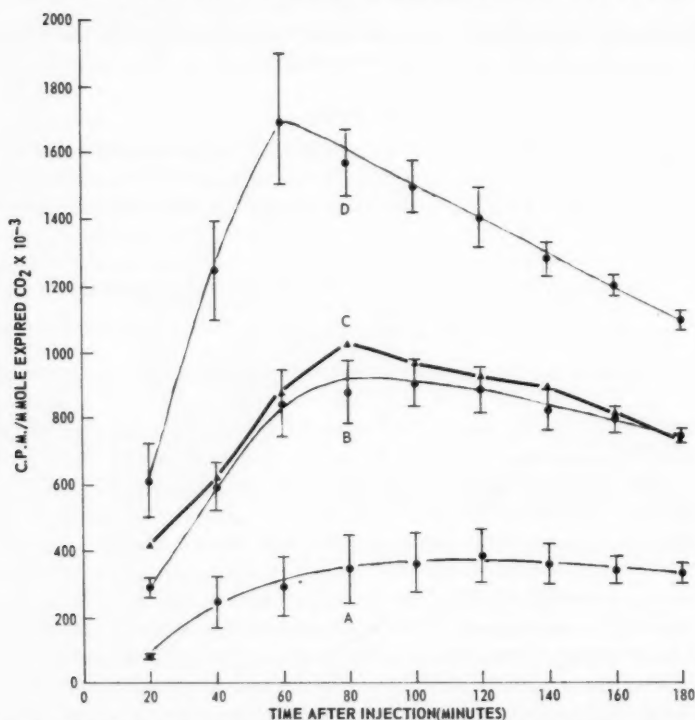


FIG. 1. Specific activity of expired CO_2 . Vertical bars indicate standard error of mean, not shown for group C. Group: A, pregnant fed; B, ketotic; C, non-pregnant fed; D, non-pregnant starved.

TABLE II
Incorporation of C^{14} into sterols of liver following intraperitoneal injection of acetate- 1-C^{14}

Group	mg AcAc/ 100 ml blood	Sterol, mg/g	% incorporation $\times 10^3$	c.p.m./mg sterol
Ketotic (10)	$11.0 \pm 1.52^\dagger$	2.71 ± 0.207	32.9 ± 10.0	847 ± 235.3
Pregnant fed (6)	<1	2.95 ± 0.059	$5.8 \pm 2.0^{**}$	$118 \pm 34.6^{***}$
Non-pregnant fed (5)	<1	2.89 ± 0.305	$7.9 \pm 1.3^*$	$251 \pm 47.3^*$
Non-pregnant starved (6)	1.8 ± 0.24	3.15 ± 0.092	11.1 ± 2.2	375 ± 60.4

NOTE: Numbers in parentheses indicate number of animals used.

Differs significantly from ketotic group where indicated:

* $P < .05$.

** $P < .02$.

*** $P < .01$.

† Standard error of the mean.

Fatty acid data presented in Tables IV and V indicate that the N.P.F. group incorporated more C^{14} into liver fatty acids than the P.F. group ($P < .02$) and that hepatic lipogenesis decreased during latter stages of pregnancy. Tracer

TABLE III
Incorporation of C^{14} into sterols of carcass following intraperitoneal
injection of acetate- $1-C^{14}$

Group	mg AcAc/ 100 ml blood	Sterol, mg/g tissue	% incorporation $\times 10^3$	c.p.m./mg sterol
Ketotic (10)	$11.0 \pm 1.52^\dagger$	$1.48^* \pm .116$	$39.7^* \pm 6.2$	$154^* \pm 31.0$
Pregnant fed (6)	<1	$1.43 \pm .137$	31.4 ± 11.9	105 ± 28.7
Non-pregnant fed (5)	<1	$1.27 \pm .080$	26.2 ± 4.2	126 ± 35.8
Non-pregnant starved (6)	1.8 ± 0.24	$1.23 \pm .058$	26.1 ± 3.1	146 ± 16.3

NOTE: Numbers in parentheses indicate number of animals used.

*Does not differ significantly from other groups.

† Standard error of the mean.

TABLE IV
Incorporation of C^{14} into fatty acids of liver following intraperitoneal
injection of acetate- $1-C^{14}$

Group	mg AcAc/ 100 ml blood	Fatty acids, mg/g	% incorporation $\times 10^3$	c.p.m./mg fatty acid
Ketotic (10)	$11.0 \pm 1.52^\dagger$	92.0 ± 12.27	14.16 ± 2.69	116 ± 16.9
Pregnant fed (6)	<1	$25.8 \pm 4.22^{**}$	7.06 ± 1.94	201 ± 57.2
Non-pregnant fed (5)	<1	$46.5 \pm 9.42^*$	29.54 ± 7.67	$625 \pm 179.4^*$
Non-pregnant starved (6)	1.8 ± 0.24	$52.5 \pm 7.08^*$	15.07 ± 3.94	$323 \pm 57.2^{**}$

NOTE: Numbers in parentheses indicate number of animals used.

Differs significantly from ketotic group where indicated:

* $P < .05$.

** $P < .01$.

† Standard error of the mean.

TABLE V
Incorporation of C^{14} into fatty acids of carcass following intraperitoneal
injection of acetate- $1-C^{14}$

Group	mg AcAc/ 100 ml blood	Fatty acids, mg/g tissue	% incorporation	c.p.m./mg fatty acid
Ketotic (10)	11.0 ± 1.52	$178.6 \pm 24.02^\dagger$	<0.2	<10
Pregnant fed (6)	<1	171.9 ± 22.51	0.86	22
Non-pregnant fed (5)	<1	149.6 ± 20.98	3.20 ± 1.19	163
Non-pregnant starved (6)	1.8 ± 0.24	$111.9 \pm 13.78^*$	0.44 ± 0.06	30

NOTE: Numbers in parentheses indicate number of animals used.

Differs significantly from ketotic group where indicated:

* $P < .05$.

† Standard error of the mean.

incorporation into carcass fatty acids (Table V) showed the expected decrease after 72 hours fasting ($P < .05$) but the incorporation in both N.P.F. and N.P.S. groups was greater than in the ketotic group. Results for the P.F. group were difficult to evaluate because of extreme variation.

Table VI shows the plasma fatty acid concentration in fasted and non-fasted pregnant guinea pigs. Non-pregnant animals fasted for 72 hours ($1.8 \pm 0.24^*$ mg acetoacetate per 100 ml blood) had $9.4 \pm 0.58^*$ meq fatty acids per liter

*Standard error of the mean.

TABLE VI
Total fatty acids in plasma of fasted and non-fasted
pregnant guinea pigs

Fasting period (hours)	mg AcAc/100 ml blood	Plasma fatty acids, meq/liter
0	0.1	6.3
0	0.1	6.7
48	1.8	259.7
24	1.9	61.4
24	2.5	240.7
48	4.1	276.7
45	4.7	49.3
48	4.9	428.1
36	6.7	236.5
48	7.7	337.7
48	10.0	11.1
57	17.4	397.3
48	25.2	3.9
48	26.4	5.9

plasma. The pregnant group included animals fasted 48 hours or less that had not yet developed ketosis in order to show that an increase in plasma fatty acid concentration may precede the onset of ketosis. This fat-mobilizing process may be temporary since it was not observed in animals with high blood acetoacetate concentration. Chernick and Scow (28) observed that diabetic ketotic rats developed hyperlipemia with increased total lipid content of liver and kidney unless the body fat stores were depleted prior to the onset of diabetes. Similarly, the hyperlipemia accompanying ketosis in guinea pigs may account for the fat accumulation in liver (Table IV). The rapid onset and relatively short duration of this fat-mobilizing process favor the possibility that it represents one of the effects of a severely challenged endocrine system.

TABLE VII
Data obtained with guinea pig liver homogenates incubated
90 minutes at 37° C in O₂

Determination	Pregnant fed group (5)	Ketotic group (5)
AcAc/100 ml blood (mg)	< 1	16.2 ± 2.38†
O ₂ uptake (μmoles)	24.9 ± 0.71**	17.1 ± 1.07
% C ¹⁴ of acetate-1-C ¹⁴ incorporated into CO ₂	20.9 ± 2.04*	12.7 ± 1.11
AcAc synthesis (μg)	118 ± 15.9	162 ± 19.9
Acetate-1-C ¹⁴ incorporated into AcAc (mμmoles)	53 ± 5.2**	17 ± 1.7
Formation of water-soluble hydroxamic acids (μmoles)	2.9 ± .26**	1.47 ± .08

NOTE: Numbers in parentheses indicate number of animals used.

Significant differences are indicated:

*P < 0.01.

**P < 0.001.

†Standard error of the mean.

Table VII shows results obtained with liver homogenates from pregnant fed and ketotic animals. The amount of tracer incorporated into CO_2 and acetoacetate, the micromoles of O_2 consumed, and the micromoles of water-soluble hydroxamic acids formed are significantly lowered in the ketotic group. Reduced O_2 consumption has also been observed in ketotic ox liver homogenates (29), alloxan-diabetic rat liver slices (30), and homogenates (31). McCarthy and Shaw (32) have suggested that hepatic metabolism is decreased in the ketotic state. The present results indicate that although incorporation of tracer into acetoacetate decreased in ketotic homogenates, acetoacetate production was not impaired. This suggests that in the ketotic liver ketogenesis is maintained in the presence of decreased metabolic activity and that more acetoacetate is derived from endogenous acetyl- or acetoacetyl-CoA than in normal liver.

Discussion

The results of these experiments indicate that fasting ketosis of guinea pigs may be accompanied or preceded by a rapid mobilization of depot fat. This fat-mobilizing process is probably mediated through the effects of a stimulated endocrine system (33) and results in an increased fatty acid concentration of the liver. A reduction in 'acetate pool' size was postulated, based on the finding that utilization of tracer acetate was not impaired in the ketotic animal while total CO_2 production was significantly decreased. This presents an apparent paradox: increased acetoacetate production in the presence of a decreased 'acetate pool' size. However, there is evidence to suggest that ketone bodies originate primarily from fatty acids of liver. Chernick and Scow (28) observed that neither ketosis nor fatty livers developed in diabetic rats that were first depleted of body fat. Thus acetoacetyl-CoA may form through oxidation of butyryl-CoA and yield acetoacetate probably either via the HMG-CoA cycle of Lynen *et al.* (34) whereby half the acetoacetate molecule may be derived from sources other than acetyl-CoA, or by direct deacylation as suggested by Drummond and Stern (35). It has already been reported (11) that in diabetic ketosis the HMG-CoA content of liver increases significantly without any apparent increase in acetyl-CoA and that the activity of acetoacetate-synthesizing enzymes is increased (10, 11). The present finding that, in ketosis of fasted guinea pigs, sterol synthesis and thus by implication acetoacetate synthesis is increased and that acetate oxidation is unimpaired supports the suggestion that ketosis is a manifestation of increased ketogenesis rather than decreased utilization of ketone bodies.

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IN VIVO INHIBITION OF CHOLESTEROL BIOSYNTHESIS BY A MITOCHONDRIAL EXTRACT¹

B. B. MIGICOVSKY

Abstract

Aqueous extracts of liver mitochondria were administered to rats by intraperitoneal, subcutaneous, and oral routes, and C¹⁴-acetate or mevalonate was injected intraperitoneally 3 hours later. Cholesterol synthesis *in vivo* from C¹⁴-acetate was depressed by the inhibitory substance in the mitochondrial extracts. Synthesis from C¹⁴-mevalonate was not inhibited.

Active extracts were prepared from livers of rat, rabbit, pig, and sheep. An inhibitory substance is present in blood serum and administration of active mitochondrial extracts depressed blood cholesterol levels. Pending its identification, the active principle has been provisionally termed I.C.S. (inhibitor of cholesterol synthesis).

Introduction

The inhibitory effect of mitochondria from livers of starved rats and of aqueous extracts of liver cell mitochondria on *in vitro* cholesterol synthesis was reported by Migicovsky (1, 2) and Migicovsky and Wood (3). It was suggested that this inhibitory substance present in the mitochondria constitutes, in part, the physiological mechanism that controls cholesterol synthesis. Bucher (4) suggested that physiological control of cholesterol synthesis operates between acetoacetate and mevalonate in the biosynthetic pathway. Shah *et al.* (5) showed that in pyridoxine deficiency where cholesterol synthesis is increased, the rate-controlling reaction occurs between acetate and mevalonic acid. Inhibition *in vitro* occurred between acetoacetate and mevalonic acid in the biosynthetic pathway. The inhibitor substance, present in mitochondrial extract, will hereafter be referred to as I.C.S., an abbreviation of "inhibitor of cholesterol synthesis". Further studies on the inhibition of cholesterol synthesis by mitochondrial extract, particularly those dealing with the *in vivo* effect, are presented in this paper.

Materials and Methods

The liver homogenate preparation of Bucher (6) was used in the *in vitro* experiments. The technique of measuring the incorporation of acetate and mevalonate into cholesterol was the same as described by Migicovsky and Wood (3).

The mitochondrial extract which contains I.C.S. was prepared from livers of rat, sheep, rabbit, and pig. The method of preparation was essentially the same in all instances and has been described by Migicovsky (2). One change introduced consisted of freezing and thawing the mitochondrial suspension prior to disruption in a 10-kc Ratheon oscillator for 8 minutes.

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Contribution No. 55, Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

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Male rats weighing between 120 and 130 g were treated intraperitoneally, subcutaneously, or orally with different quantities of I.C.S. dissolved in 1 ml of saline. Control rats of the same weight and sex were treated with isotonic saline. Three hours after injection all rats were given an intraperitoneal dose of C^{14} -acetate. Two hours after the acetate injection the rats were killed by decapitation and the livers were excised and homogenized in water. An aliquot of the liver homogenate was saponified and extracted with petroleum ether, and cholesterol digitonide was prepared as described by Migicovsky (1).

Determination of rabbit serum cholesterol was carried out by the method of Crawford (7) and liver cholesterol was determined by the Lieberman-Burchard reaction as described by Cook (8).

Radioactivity measurements were made with a gas flow counter and appropriate corrections for pad thickness were applied. Cholesterol was counted as the digitonide and reported in terms of cholesterol.

Results

In Vitro Experiments

Extracts of mitochondria ($9,000\times g$ sediment) and microsomes ($100,000\times g$ sediment) prepared from the same rat liver were tested for inhibitory activity on the same homogenate preparation. Results in Table I illustrate that the microsomal extract was inactive, and that inhibitory activity was confined to mitochondria.

TABLE I
In vitro activity of mitochondrial
and microsomal extracts

Addition to homogenate*	μ moles acetate incorporated $\times 10^3$
None	3.12
2 mg mitochondrial extract	1.36
2 mg microsomal extract	3.10

*Substrate was 1.31 μ moles C^{14} -acetate which had 4×10^4 c.p.m.

Table II shows results of experiments in which mitochondrial extracts prepared from livers of three species of animal, namely sheep, pig, and rabbit, were tested for inhibition of cholesterol synthesis from acetate by a Bucher homogenate of rat liver. The data illustrate that the lyophilized aqueous extract of liver mitochondria from all species contained I.C.S.

It is appreciated that in vitro activity of a substance does not necessarily reflect its physiological activity in the intact animal. This was clearly demonstrated by Wood and Migicovsky (9, 10), who found that unsaturated fatty acids inhibited cholesterol synthesis in vitro but promoted synthesis in vivo.

In Vivo Experiments

Inhibitory activity of pig liver mitochondrial extract, crude as it is, was tested on intact rats and results of the first trial in which I.C.S. was injected

TABLE II
In vitro activity of I.C.S. from liver of different species

I.C.S. added to homogenate* (mg)	μ moles acetate incorporated $\times 10^3$		
	Sheep	Pig	Rabbit
None	7.08	2.86	2.87
1	5.49		
2	4.86	1.02	
4	0.62	0.48	0.10
6	0.17	0.22	0.02

*Substrate was 1.31 μ moles C^{14} -acetate which had 4.77×10^4 c.p.m.

intraperitoneally are shown in Table III. Values for the specific activity of the liver cholesterol obtained for the I.C.S.-treated rats are lower than for the controls, indicating that cholesterol synthesis from acetate was inhibited.

TABLE III
Effect of an intraperitoneal injection of
pig I.C.S. into rats*

Treatment	c.p.m./mg liver cholesterol
50 mg I.C.S.	886
100 mg I.C.S.	982 (919 \pm 31.3)†
150 mg I.C.S.	890
Saline	1100
Saline	1100 (1131 \pm 31.3)
Saline	1194

*All rats received intraperitoneally 6.55 μ moles C^{14} -acetate containing 23.8×10^4 c.p.m. 3 hours after the injection of mitochondrial extract or saline.

†Mean and S.E._{mean}.

Table IV shows the effect of a subcutaneous injection of pig I.C.S. In this experiment the I.C.S. dose was divided into three doses of 200 mg each and was given over a period of 18 hours to each of four rats. Four control rats received a saline injection. Three hours after the last dose 3.32 μ moles of C^{14} -

TABLE IV
Effect of a subcutaneous injection of pig I.C.S. into rats*
(three doses of 200 mg/dose = 600 mg)

c.p.m./mg liver cholesterol	
Treated	Untreated (saline)
2100	5753
2562	3338
2110	3568
2012	3316
(2196 \pm 124)†	(3994 \pm 589)

*All rats received subcutaneously 3.32 μ moles C^{14} -acetate containing 28.63×10^4 c.p.m. 3 hours after the injection of I.C.S.

†Mean and S.E._{mean}.

acetate was injected intraperitoneally. The results indicate that subcutaneous injection of I.C.S. inhibited synthesis of liver cholesterol *in vivo*.

The results of several trials conducted at different times are shown in Table V together with the amounts of I.C.S. and of acetate injected. The results on all trials were consistent in demonstrating an inhibitory effect on cholesterol synthesis. The variation in specific activity of the cholesterol between trials conducted at different times is quite large: it is difficult to avoid or circumvent this variation since many factors, including physical environment, can alter the rate of cholesterol synthesis. When the factors are the same for both treated and control rats the treatment exerts a significant effect on cholesterol synthesis.

TABLE V
Effect of an oral dose of pig I.C.S. into rats*

Trial No.	c.p.m./mg liver cholesterol	
	Treated	Untreated
1 (150 mg)	960	2576
	476	2024
	852	1760
	(763 ± 147)†	(2120 ± 240)
2 (250 mg)	1318	1430
	1280	1888
	1290	1716
	1242	1894
	(1282 ± 16)	(1732 ± 109)
3 (250 mg)	780	928
	586	836
	693	880
	424	884
	(621 ± 59)	(882 ± 19)

*Rats in trials 1 and 2 received orally 6.55 μ moles C^{14} -acetate containing 23.67×10^6 c.p.m. and rats in trial 3 received 6.99 μ moles C^{14} -acetate containing 31.83×10^6 c.p.m. 3 hours after dose of I.C.S.

†Mean and S.E._{mean}.

Since it has been shown that I.C.S. inhibits *in vitro* synthesis when the substrate is acetate but not when it is mevalonic acid, an experiment was conducted to test this fact *in vivo*.

Sixteen male rats weighing between 125 and 135 g were treated as shown in Table VI and the specific activity of the liver cholesterol was measured. The results indicate that a single oral dose of 500 mg of pig I.C.S. inhibited synthesis of cholesterol from acetate but not from mevalonate.

The specific behavior of I.C.S. with respect to substrate strengthens the argument that it has a specific role to play in the physiological control of cholesterol synthesis.

In Vitro Assay of I.C.S. in Serum

Does I.C.S. produced in the mitochondrion find its way into the general circulation? Tables VII and VIII show the results of tests for inhibitory

TABLE VI
Effect of an oral dose of 500 mg of pig I.C.S. into rats*

Substrate	c.p.m./mg liver cholesterol	
	Treated	Untreated
¹⁴ C-Acetate	912	2532
	1500	2008
	1516	2372
	1056	2168
	(1246 ± 154)†	(2270 ± 115)
¹⁴ C-Mevalonate	7180	6192
	6940	5644
	7320	7528
	6252	7248
	(6923 ± 237)	(6653 ± 443)

*Dose of ¹⁴C-acetate was 3.32 μ moles containing 26.73×10^4 c.p.m. Dose of ¹⁴C-mevalonate was 4.08 μ moles containing 5.12×10^4 c.p.m.

†Mean and S.E.-mean.

activity of sera from rats and humans. It is apparent that the sera do contain an inhibitor. Further studies should ascertain if the inhibitor in serum is the same as that produced by mitochondria.

TABLE VII
In vitro inhibitory activity of rat serum

Addition (ml)	μ moles acetate incorporated $\times 10^3$
None	8.05
0.1	6.56
0.2	3.35
0.3	2.38

1.03 μ moles ¹⁴C-acetate containing 2.28×10^4 c.p.m. was used as the substrate.

TABLE VIII
In vitro inhibition of cholesterol synthesis by human serum

Addition to homogenate	μ moles acetate incorporated $\times 10^3$
None	17.98
4 mg pig I.C.S.	3.23
0.2 ml serum No. 1	5.59
0.2 ml serum No. 2	5.28
0.2 ml serum No. 3	4.34
0.2 ml serum No. 4	7.85

0.665 μ mole ¹⁴C-acetate containing 5.66×10^4 c.p.m. was used as the substrate.

Effect on Cholesterol Level of Blood Serum

The effect of I.C.S. on cholesterol level of serum has been studied with rabbits. In the first experiment two rabbits were used. Rabbit No. 1 was given 500 mg of pig I.C.S. intravenously. Time of injection is referred to as

TABLE IX
Blood cholesterol in rabbits

Treatment	Time (hour)	mg cholesterol/ 100 ml serum
Rabbit No. 1		
500 mg pig I.C.S. (I.V.)	0	94.9
	3	65.4
	6	59.2
	12	55.0
	24	49.9
Saline (I.V.)	48	74.1
	51	75.2
	54	75.2
	60	76.5
	72	74.0
Rabbit No. 2		
Saline (I.V.)	0	70.3
	3	67.8
	6	66.0
	12	69.1
	24	71.5
500 mg pig I.C.S. (I.V.)	48	87.6
	51	78.9
	54	76.5
	60	64.1
	72	77.7

TABLE X
Blood cholesterol in rabbits

Treatment	Time (hour)	mg cholesterol/ 100 ml serum
Rabbit No. 1		
200 mg rabbit I.C.S. (I.V.)	0	85.7
	3	56.3
	6	42.9
	12	49.1
	30	87.5
Saline (I.V.)	54	90.0
	57	86.3
	63	90.0
	75	85.4
	99	85.4
	147	97.5
Rabbit No. 2		
Saline (I.V.)	0	75.0
	3	75.9
	6	74.1
	12	78.6
	30	74.1
200 mg rabbit I.C.S. (I.V.)	54	77.9
	57	59.4
	63	49.2
	75	55.7
	99	68.7
	147	87.2

TABLE XI
Blood cholesterol in rabbits

Time (hour)	Rabbit No. 1		Rabbit No. 2		Rabbit No. 3	
	Treatment	mg cholesterol/ 100 ml serum	Treatment	mg cholesterol/ 100 ml serum	Treatment	mg cholesterol/ 100 ml serum
0	1 g pig I.C.S. (oral)	179				
24	1 g pig I.C.S. (oral)	148				
30		109	500 mg pig I.C.S. (I.V.)	171	Saline (I.V.)	150
36		124		137		147
48		130		94		145
				119		157
96		168		167		155

time zero. Blood was sampled from the ear vein at various intervals thereafter. Forty-eight hours after I.C.S. injection, the rabbit was given an intravenous injection of isotonic saline. Rabbit No. 2 was treated the same as No. 1 except that the initial injection at zero time was saline and 48 hours after the saline injection an intravenous dose of 500 mg of pig I.C.S. was administered. The results of the serum cholesterol levels, as determined by the method of Crawford (7), are shown in Table IX.

The second experiment (Table X) was carried out in the same manner as above except that 200 mg of rabbit I.C.S. was injected.

The third experiment utilized three rabbits (Table XI). Rabbit No. 1 was given two oral doses of 1 g each of pig I.C.S. and blood was sampled at intervals after the second dose. Rabbit No. 2 was given a single intravenous injection of 500 mg of pig I.C.S. and the third rabbit received isotonic saline.

Results of these rabbit experiments indicate that I.C.S. treatment depresses serum cholesterol within a very short time. The single case which did not respond was rabbit No. 2 in the second experiment.

Results of these experiments are insufficient to permit generalization that I.C.S. will reduce serum cholesterol levels. They do indicate that further experimentation with I.C.S. along this line is desirable.

Conclusions

An aqueous extract of mitochondria, previously shown to inhibit *in vitro* cholesterol synthesis from acetate, has been found to have similar inhibitory properties *in vivo* when administered intraperitoneally, intravenously, or orally. Preliminary experiments indicate that the inhibitory factor, provisionally termed I.C.S., may be effective in reducing the level of serum cholesterol. It has been shown that rat and human sera contain a substance, or substances, which inhibit cholesterol synthesis from acetate *in vitro*. It is possible that the substance in serum is the same as that obtained from liver mitochondria.

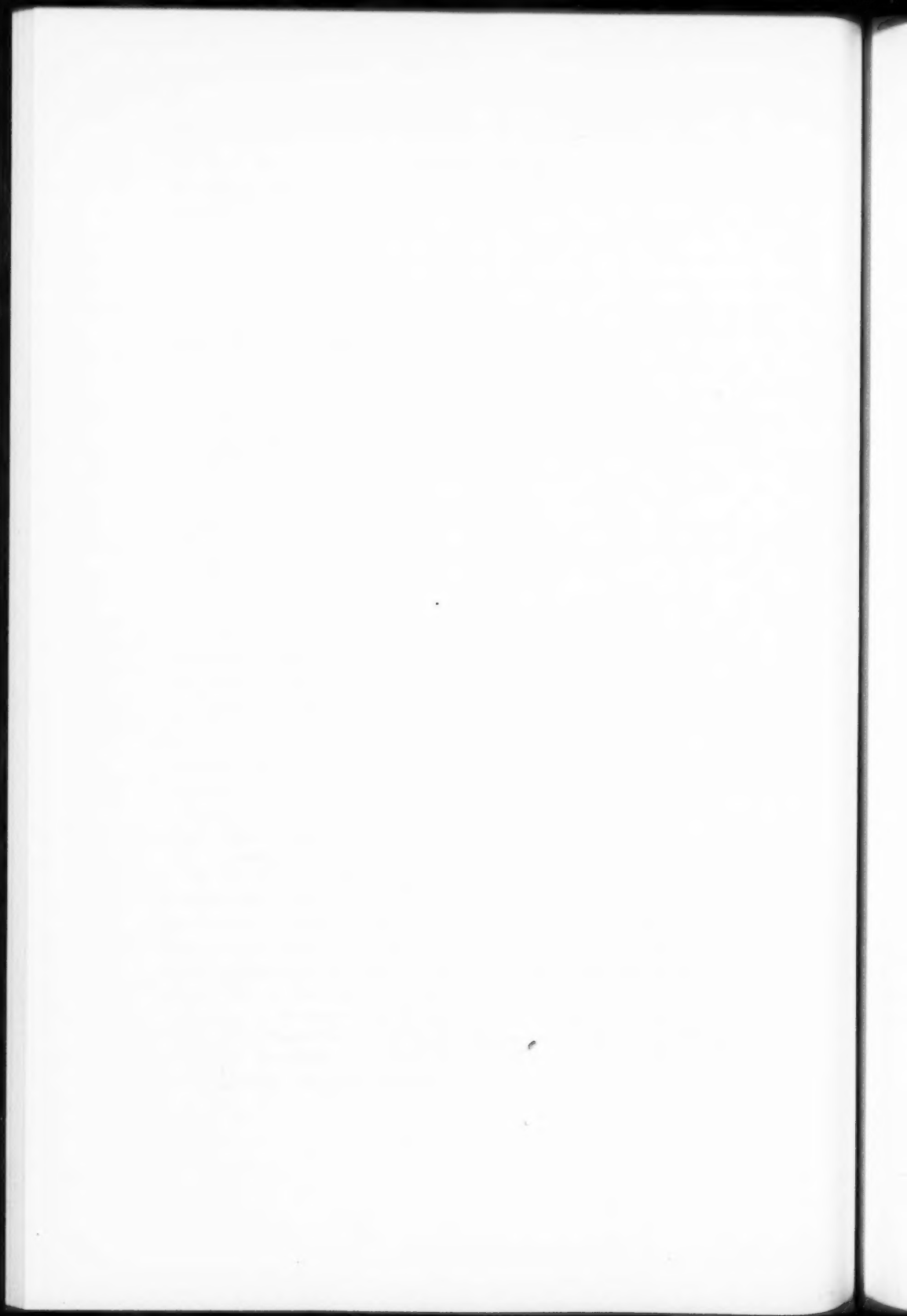
The I.C.S. substance shows promise of being the principle which is, in part, responsible for regulating cholesterol synthesis. Although it is found in the mitochondrial fraction, an earlier experiment described by Migicovsky and Wood (3) demonstrated its presence in the soluble portion of a Bucher (6) homogenate. When the clear supernate of a rat liver homogenate, capable of synthesizing cholesterol from acetate, is replaced by the clear supernate from a starved rat liver homogenate, the degree of synthesis is reduced by 70%. A similar experiment described by Bucher (4) illustrates the depressing effect of the soluble fraction from livers of starved rats. It is believed that a substance, inhibitory to cholesterol synthesis, is present in the mitochondrial fraction of liver and is released at a variable rate depending on the physiological stimulus. Starvation provides a stimulus which brings about increased release of this substance from the mitochondrion.

Acknowledgments

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EFFECTS OF LIPID MATERIALS ON GUINEA PIG COMPLEMENT¹

R. K. GUTHRIE, J. R. DOWDY, AND D. C. HINKLE

Abstract

Prior work has shown that the fourth component of guinea pig complement is increased following antigen injection. This report concerns the changes in C'4 following injection of non-antigenic materials. Injection of cholesterol, ergosterol, or linoleic acid does not cause any measurable change in C' or C'4. Injection of oleic or of stearic acid causes a significant increase in C'4 but has no effect on C'.

Introduction

Recently it has been shown (1) that C'4 in guinea pig and rat serum is increased following antigen injection. That such an increase may be correlated with antibody response following antigen injection was demonstrated in work (2) showing that the simultaneous injection of antigen and corticosteroids caused a reduction in the level of this complement component.

With these suggestions that the increase in C'4 may be a part of the immune response of the animal, it becomes desirable to learn if other, non-antigenic materials can cause such an increase. Prior reports of administration of such chemicals as cholesterol, ascorbic acid, and biotin (3, 4, 5, 6) have shown that these chemicals have essentially no effect on the hemolytic activity of complement in guinea pigs. Since, in much of the work described, total hemolytic activity of all components present was tested with no titrations performed to determine the level of the individual components, it must be considered that such reports deal with the component naturally present in smallest amount (in guinea pig serum C'2) and that such titrations will not determine significant effects on excess components. Boulanger *et al.* (9), in studying complement components, noted a decline in C'2, C'3, and C'4 in guinea pigs during pregnancy, at about the same period that increases in plasma lipids had previously been reported. These workers also reported a reduction in all components in animals treated with dibestranol and (or) progesterone. Rice *et al.* (10) found decreases in C'2 and C'3 following treatment with carbon tetrachloride but no appreciable changes in C'1 or C'4. Ethionine had a marked reducing effect on all components of guinea pig complement. All titers returned to normal after cessation of treatment. Rice *et al.* (11) found also that chloroform anaesthesia reduced complement titers in guinea pigs, the reduction being primarily in C'1 and C'2. Since there are reports (7) that complement titers of rabbits and rats could be affected by various chemical additions to the diet or chemical injections, it seems likely that these chemicals may have an

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effect on components other than C'2, which is not necessarily the lowest component in these animals. Rice *et al.* have stated that all components of rabbit complement are reduced following ethionine treatment, as was seen in the guinea pig (10).

The present paper deals with an attempt to determine the effect, on C'4 titers in the guinea pig, of injection of lipid materials. Cholesterol, ergosterol, and oleic acid were chosen because of their presumed non-antigenic nature and because they represented different types of lipid materials. Of these, cholesterol and ergosterol have been reported (3) to have no specific effect on total hemolytic activity of complement in the guinea pig, but the study did not include the effect on the component present in largest amount in the guinea pig, C'4.

Materials and Methods

Complement Titration

Complement titrations were carried out using a procedure modified from Kabat and Mayer (see reference 1); the results are given in 50% hemolytic units as calculated from the table of Kabat and Mayer (8). In the work reported in reference 1, titers were lower than in the present work. This variation is not due to a difference in calculation, but to an improvement in technic (i.e. in the form of water used in preparation of saline) which permitted higher dilutions of serum and more accurate detection of alterations in titer.

Test Animals

Young guinea pigs weighing under 400 grams were used in this work. The animals were housed in groups of four, on a constant diet and care schedule, and were bled from the heart, 2 ml of blood being removed for a single titration. The blood was allowed to clot and the serum was separated and titrated for C' and C'4 activity within 6 hours after bleeding. These titrations were repeated before any materials were injected into the animals. The two titrations prior to injection were averaged, and the mean is reported as the preinjection titer. The animals were then divided into control groups and test groups for each of the materials to be tested. In the control group the animals received injections of 5% ethanol, which was used as a solvent for the test chemicals. In the results, each test group was compared with the ethanol-injected control group of animals.

Injections

Following the two preinjection titers, the test animals were given, at 2-day intervals, a total of three injections of the test materials. Each test material was injected in a solution in 5% ethanol, in the following amounts: oleic acid, 9.6 mg; cholesterol, 5 mg; ergosterol, 5 mg. A second series of animals were given three injections daily, titered after 1 week, and then given a second series of three daily injections.

Following the initial test, when it was seen that alternations in the titer of C'4 occurred subsequent to injection of oleic acid in the guinea pig, a separate

series of tests was carried out in an attempt to determine the specific effect of oleic acid, linoleic acid, and stearic acid. This plan provided an opportunity of testing the effect of the degree of saturation of the 18-carbon molecule on the response observed in the animal. In test procedures involving linoleic and stearic acid, the animals used, titration procedures, and injection schedules were the same as those used in the previous tests. Amounts of materials injected had to be reduced by a factor of 4 because of the solubility of these chemicals in ethyl alcohol.

Two weeks after the first injection of the test materials, animals were again bled from the heart and complement activity and C'4 titer were measured. This titration was repeated in 4 days, the average of these titrations being reported as the postinjection titers.

Results

Results of the first series of the tests are seen in Table I. In this table the preinjection titers and postinjection titers of test animals are compared to pre- and post-injection titers of control animals. Controls received injections of 5% ethanol. In the table it is seen that there are no significant changes in the total hemolytic activity of complement (C') in any group of animals, test or control. These results support previous reports that these materials do not specifically alter the total hemolytic activity of complement. No significant change was observed in the C'4 titers of cholesterol- or of ergosterol-injected test groups.

TABLE I
Mean complement titers in test and control guinea pigs*

Animals		Time			
		Before injection		2 weeks after injection	
Treatment	Number	C'	C'4 (SD)	C'	C'4 (SD)
(1) Oleic acid†					
Control	5	258	6393 (168)	280	6486 (288)
Test	12	258	6454 (354)	270	7238 (633)
(2) Cholesterol‡					
Control	3	198	4364 (232)	200	4493 (175)
Test	9	192	2823 (896)	180	2851 (925)
(3) Ergosterol§					
Control	4	224	4324 (545)	223	4898 (944)
Test	8	209	3709 (442)	202	4206 (754)

*All test and control animals were selected arbitrarily before titer from lots of guinea pigs received in one shipment. Such selection accounts for initial titer variations. All mean titers represent two or more titrations of serum obtained at 4-day intervals.

†Nine test animals showed an increase after oleic acid injections, with six of these increases being greater than one standard deviation.

‡Five test animals showed increases after cholesterol injections, with no increase being as great as one standard deviation.

§Six test animals showed increases after ergosterol injections, with no increase being as great as one standard deviation.

In the oleic acid animals, while the control group shows no significant change in C'4, the test group shows an increase of 784 units, 13%. Statistical analysis of this change in titer shows a p value of 0.001 for the observed increase in titer. The apparent statistical significance here is accounted for in the fact that in six of the nine experimental animals receiving oleic acid the increase was more than one standard deviation; in only two was there a decrease, and this was less than one standard deviation.

The increase in titer is approximately the same in degree and significance, as was previously shown in guinea pig serum following injection of antigens (1). Following observance of this increase it was necessary to determine whether there is any specific effect of oleic acid itself, or whether this same effect could be observed by the use of similar chemical compounds, varying only in the saturation of the carbon chain of the fatty acid.

The results for the second series of tests are reported in Table II. In this series it is seen again that the control animals tested without injection over the same time period show essentially no change in titer, either of C' or C'4. It is

TABLE II
Mean complement titers in test and control guinea pigs*

Animals		Time				
		Before injection			2 weeks after injection	
		C'	C'4	(SD)	C'	C'4 (SD)
(1) Oleic acid†						
Control	6	323	8342	(860)	331	8000 (928)
Test	6	277	7183	(816)	279	10475 (923)
(2) Linoleic acid‡						
Control	6	256	6209	(963)	234	6621 (1060)
Test	6	256	6680	(843)	264	5457 (536)
(3) Stearic acid§						
Control	5	279	7111	(1350)	240	6620 (1181)
Test	5	274	8289	(413)	256	9000 (580)

*All test and control animals were selected arbitrarily before titer from lots of guinea pigs received in one shipment. Such selection accounts for initial titer variation. All mean titers represent three or more titrations of serum obtained at 4-day intervals.

†Five test animals showed an increase after oleic acid injections of more than one standard deviation, while the sixth showed no change.

‡One animal showed an increase of more than one standard deviation and three showed a decrease of more than one standard deviation, following linoleic acid injection.

§All test animals showed an increase of more than one standard deviation following stearic acid injection.

also observed here that injection of any one of the acids has no effect on the C' titer in the test animals. Injection of linoleic acid actually shows a decrease in C'4 titer, although it is of doubtful significance. Injection of oleic acid resulted in an increase of 3292 units, a 46% increase. When statistically examined this change has a p value of 0.001. Injection of stearic acid induces a smaller increase in titer of C'4 than does the injection of oleic; however, the total amount

of stearic acid injected was less than that of oleic because of the solubility of the acids in ethyl alcohol. The increase in C'4 titer in the stearic acid tests, although of smaller total amount, shows a *p* value of 0.001 when statistically analyzed.

In a prior report (1), it was seen that the titer of the C'4 component of complement remained essentially unchanged in adult guinea pigs for several weeks when the animals were not injected with any material. In Table III are

TABLE III
Maintenance of elevated C'4 titer in oleic acid injected guinea pigs*

Animal number	Pre-injection titer	Date	Post-injection titer (1)	Date	Post-injection titer (2)	Date
1	6630	8-5-60	5685	8-18-60	5685	10-28-60
2	5685	8-5-60	6915	8-18-60	7500	10-28-60
3	7500	8-9-60	7500	8-23-60	8130	10-28-60
4	6915	8-9-60	9560	8-23-60	9345	10-28-60
5	7855	9-9-60	10445	9-28-60	11640	10-28-60
6	6330	9-9-60	11640	9-28-60	11640	10-28-60

*Titers reported are mean titers representing at least two titrations of serum taken at 4-day intervals. Dates listed are the last titration date for that period.

results indicating that, following oleic acid injections, the C'4 titer increases within a 2-week period, and that this titer remains high for up to 3 months following the injections. The only one of six animals not maintaining a high titer for this period was the one guinea pig which did not show an increase within 2 weeks. The tests shown in these results were on animals receiving the oleic acid injection schedule shown in the other tables.

Discussion

Injection of a group of lipid materials into the guinea pig does not have any measurable effect on the total hemolytic activity of guinea pig complement. This finding supports previously reported results with similar materials.

Injection of oleic, and of stearic, acid into the guinea pig is followed within 2 weeks by an increase in the titer of C'4. The increase appears greater in the case of oleic acid injection. However, this difference in degree is perhaps due to the difference in amount of material injected. In either case the results are significant when statistically analyzed, having a probability of 0.001.

In testing animals for the possibility that these materials acted as antigens in producing this increased C'4, emulsification of fatty acids, and attempts to conjugate these acids with protein, gave no evidence in vitro of a precipitation reaction with serum from injected guinea pigs. Emulsification and injection of the fatty acids produced no in vivo symptoms of anaphylactic shock. It seems likely from these results that some mechanism other than antigenic stimulation was responsible for the increased C'4.

An earlier report (1) showed that uninjected animals maintained an essentially constant C'4 titer over a period of several weeks and indicates that

the C'⁴ titer in the guinea pig is relatively stable, except for seasonal variations (13). The results in Table III indicate that where an increase results from oleic acid injection this increased titer is also maintained for several weeks; the animals maintained or increased their C'⁴ titers for from 6 weeks to 3 months following the injections. These results would suggest that the increase is due not only to an immediate release of the C'⁴ component from the tissues but to the fact that this serum component continues to be released at the increased rate following injection of oleic acid.

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THE EFFECT OF INTRAVASCULAR PRESSURE CHANGES ON BLOOD FLOW¹

THOMAS ZSOTÉR

With the technical assistance of J. LIEBMAN AND G. CHARBONNEAU

Abstract

The sudden intra-arterial injection of saline, Locke's solution, or blood, at different speeds into the arteries of dogs, caused an increase in blood flow at the site of injection and distal to it. The decrease in the vascular resistance, produced by this stretching, was more marked in the femoral than in the carotid artery of the dog. Intact vascular innervation is not required to obtain this effect. The results suggest that the vascular response to stretching, used in these experiments, would not be simply a passive one. The results do not support the myogenic theory of Bayliss and of Folkow.

1. Introduction

The relation between pressure and flow is a well-studied aspect of peripheral circulation. The effect on the blood flow of a sudden intravascular pressure increase, i.e., of arterial stretching, however, is not so well established. Bayliss (1) and Folkow (2, 3) claim that a decrease of intravascular pressure results in vasodilation, whilst an increase of pressure results in vasoconstriction. This was attributed to a myogenic response. However, we have obtained some results in our experiments on the effect of sudden injection of physiological saline on the blood flow, which seemed to us hardly compatible with the findings of Bayliss and Folkow. For this reason this problem has been studied in greater detail.

2. Methods

Ninety-six mongrel dogs, weighing 6.1–15.0 kg, anaesthetized with sodium pentobarbital (20 mg/kg i.v., 30 mg/kg i.m.) were used in these experiments. Blood clotting was prevented by injection of 6 mg/kg heparin (1 mg 134 U.S.P. units).

In one group of dogs, blood flow and arterial pressure were determined by Girling's method (4). By this method the artery is doubly cannulated. The proximal cannula leads into a small chamber separated from an ink-writing mercury manometer by a slack rubber membrane, while the distal cannula leads from the chamber back into the artery. When the proximal plastic cannula is occluded for a short period (for 2 seconds in our experiments), the pressure in the manometer, transmitted through the rubber membrane, will cause the blood in the chamber to flow back into the artery at a rate depending on the peripheral resistance of the limb. This will result in a rate of fall in the

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Contribution from the Ayerst Research Laboratories, Montreal, Que.

recorded pressure proportional to the blood flow. The blood flow can be calculated from the magnitude of the pressure drop, the cross-sectional area of the manometer tube, and the duration of occlusion. As the blood pressure is simultaneously registered, the local vascular resistance also can be estimated. The injections of physiological saline, Locke's solution, or blood were given manually through a T tube inserted into the distal plastic cannula, separated from the artery by a "dead space" containing at least 3 ml.

In another group of animals blood flow was registered by venous-occlusion plethysmography on a Sanborn Twin Viso Cardiette. The hind limb was inserted into the plethysmograph below or above the knee, depending on whether flow in areas containing mostly skin or mostly muscle was to be measured. In these dogs, physiological saline was injected, 2 ml over 5 seconds, directly into the femoral artery through a needle previously inserted into this vessel. Flow was measured distal to the point of injection. The vascular innervation was intact.

In order to measure the blood pressure during and following injection of saline distal to the site of injection, while blood flow was being measured by Girling's method, a Sanborn electromanometer was used, connected to a needle inserted in the distal part of the cannulated femoral artery, about 1 cm from the cannula.

In order to imitate Bayliss's experiments on the blood flow responses to intravascular pressure fall in dogs, the volume changes of the hind limb were recorded using plethysmography, without venous occlusion. The femoral arterial pressure was simultaneously recorded from the other leg using an ink-writing manometer. A sudden drop in blood pressure was produced by electrical stimulation of the cervical vagosympathetic trunk. The stimuli were square waves of 20-millisecond duration and 20/second frequency, continued for 10-60 seconds at 0-14 volts.

3. Results

(a) *Saline Injections*

Injections of 2 ml physiological saline of 5-second duration into the femoral artery, or more exactly into the dead space of the cannulating tube leading to the artery, consistently resulted in an increase of arterial inflow, estimated by Girling's method. Maximal change in blood flow, as a rule, occurred within 20 seconds after the administration of saline, and the flow remained above the control level for over 1 minute. Average flow in the first minute (Table I) was higher in 31 out of 35 experiments ($t = 4.30$, $p \ll 0.01$). The increase, expressed as percentage of control values, was generally greater, the smaller the control value. During the next few minutes blood flow returned to the preinjection level, or to a value slightly below it. Blood pressure in the femoral artery became slightly lower in all the experiments for a short period after the saline injection; this means that there was a greater decrease in local peripheral resistance than indicated by the flow changes alone. When the temperature of

the injected saline was raised to 37° instead of at room temperature, there was no difference in the above-described changes.

When physiological saline was replaced by Locke's solution, no material difference was observed in the results (Table I).

(b) Role of Viscosity of the Injected Fluid

Blood (2 ml), withdrawn from the contralateral femoral vein and injected during a period of 5 seconds, resulted in a similar effect on flow (Table I) and arterial pressure as did physiological saline. The increase in circulation was slightly higher with blood injection than with saline.

(c) Effect of Speed of Injection

Table I gives the results when the time of injection was changed, saline or Locke's solution being given in 1 second, instead of 5 seconds, and when the volume of injection was increased to 5 ml from 2 ml. Neither the maximal nor the average increase in flow in the first minute was significantly enhanced when saline or Locke's solution was given in 1 second instead of 5 seconds. On the other hand the increase in blood flow was definitely more when the greater volume, 5 ml instead of 2 ml, was injected.

In order to confirm our results, a different method, i.e. that of venous occlusion plethysmography, was used. The effect of a sudden saline injection, 2 ml in 5 seconds, on the blood flow was recorded. The maximal increase in blood flow, based on 28 observations in 21 dogs, was from 8.26 before injection to 12.52 ml/minute/100 ml tissue after injection, i.e., an average increase of 51.5%. Average flow during the first minute following the injection became 9.33 ml, a value significantly higher than the control ($t = 2.16$, $p = 0.01-0.02$). The increase of flow lasted longer in these experiments than in the previous ones, where it was recorded at the site of the injection by Girling's method. When the change in flow is expressed as a percentage increase of the control value, the effect of the injections of saline was greater in the "muscle" than in the "skin" areas.

(d) Carotid Artery

To learn how pertinent our findings would be to other vascular areas, we examined the effect of injections of saline and of blood on the blood flow of the carotid artery. In the same six dogs, blood flow response to these injections was determined both in the femoral and the carotid arteries by Girling's method. In our experiments the injections of 2 ml physiological saline in 1 second or 2 ml blood in 5 seconds consistently resulted in less increase of blood flow in the carotid than in the femoral artery of the same animal. The maximal or the average increase in the first minute was less than one third of that found in the femoral artery.

(e) Rise of Pressure upon Injection

Blood pressure in the cannulated femoral artery was registered distal to the site of saline injection in eight dogs, on 13 occasions. In all cases an injection of

TABLE I
Effect of intra-arterial injections on the femoral blood flow in dogs

Injection into the femoral artery	Number of:		Blood flow in ml/min					Average of the individual maximal changes expressed in %
	Dogs	Experiments	Maximal effect	Average flow				
				0-1 min	1-3 min	3-5 min		
2 ml physiological NaCl in 5 sec	29	35	16.8	21.2	18.1	16.7	16.2	+34.9
2 ml Locke's solution in 5 sec	11	11	17.3	21.6	18.6	16.8	16.3	+34.3
2 ml blood in 5 sec	26	31	14.1	19.2	16.4	14.1	13.7	+40.0
2 ml physiological NaCl in 1 sec	21	21	15.1	20.0	16.6	15.5	15.2	+37.8
2 ml Locke's solution in 1 sec	11	11	16.5	20.9	18.6	16.5	15.6	+34.8
5 ml physiological NaCl in 5 sec	10	10	18.0	25.9	21.3	18.4	17.5	+54.0
5 ml physiological NaCl in 1 sec	10	10	21.4	29.5	24.9	23.0	21.9	+46.2
5 ml Locke's solution in 5 sec	11	11	17.3	25.8	21.6	17.7	17.0	+76.4
5 ml Locke's solution in 1 sec	9	9	16.3	23.8	18.6	15.3	14.1	+67.9

NOTE: *t* test gave a *p* value <0.01 between 0 and 1 minute and control blood flow values in each group, except when Locke's solution was injected in 1 second; *p* was then equal to 0.02-0.05.

2 ml physiological saline, over 5 seconds, produced a rise in "local" blood pressure, on the average from 89.8 to 119.0 mm Hg. (Diastolic pressure determinations were inaccurate during the injections.) The rise in pressure occurred immediately upon injection, as in the case illustrated in Fig. 1, and returned to control value, or below, within 10 seconds. Thus, the increase of blood flow outlasted the pressure increase, usually by more than 40 seconds.

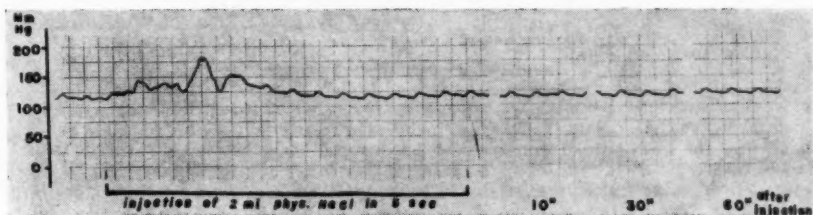


FIG. 1. Blood pressure in the femoral artery before, during, and after the injection of 2 ml saline intra-arterially. Arterial pressure was registered distal to the site of the injection.

(f) *Effect of Blocking Agents*

To study the mechanism of action of the saline injection on blood flow, such an injection was preceded by the infusion of Novocain to block the vascular nerve endings, or of iodoacetic acid (IAA) to inhibit the metabolism of the vessel musculature (5), thereby blocking the "active" participation of the vessels in blood flow changes after NaCl. In these experiments we first measured, using Girling's method, the effect of 2 ml saline and of 2 ml blood. Then either 2% procaine hydrochloride or 2% IAA was infused into the femoral artery for 10 minutes at a rate of 1 ml/minute, and the effects of saline and blood were then tested again. Immediately after Novocain the blood flow was higher and the effect of saline and blood injection slightly, but not significantly, less than previously. The maximal flow increase following the injection of 2 ml blood, expressed as percentage, became +29.5% instead of +38.7%, the average value found before Novocain infusion. One hour after Novocain infusion, the flow and the flow response to saline and blood injections were essentially the same as before Novocain.

IAA had a most striking action. The increased flow, as a result of the sudden injection of saline or blood, was almost completely abolished following the infusion of IAA (Fig. 2). Increases in flow in the first minute after blood (and saline), before and following IAA, were significantly different ($t = 3.00$, $p = 0.01-0.02$). Tests repeated 30 and 60 minutes after IAA infusion indicated a certain trend to return to the "normal" saline or blood effect.

(g) *Repetition of the Experiments of Bayliss*

A fall in the arterial pressure was produced in dogs by the electrical stimulation of the cervical vagosympathetic trunk. The return to normal values of the arterial pressure, in our experiments as in those of Bayliss, was consistently

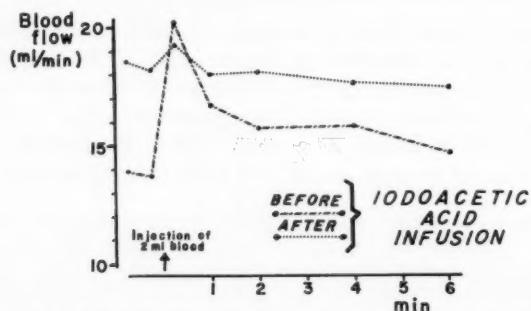


FIG. 2. The effect of the injection of 2 ml blood intra-arterially on the blood flow before and after the intra-arterial infusion of 2% iodoacetic acid. Average of experiments in seven dogs.

followed by an increase in the volume of the leg, measured by plethysmography. The increase in volume, however, in all but 2 out of 66 tests carried out in seven dogs, followed and never preceded the increase in blood pressure. Varying the intensity of the electrical stimulation, we produced two kinds of arterial pressure changes: in the first type a slow fall in blood pressure was

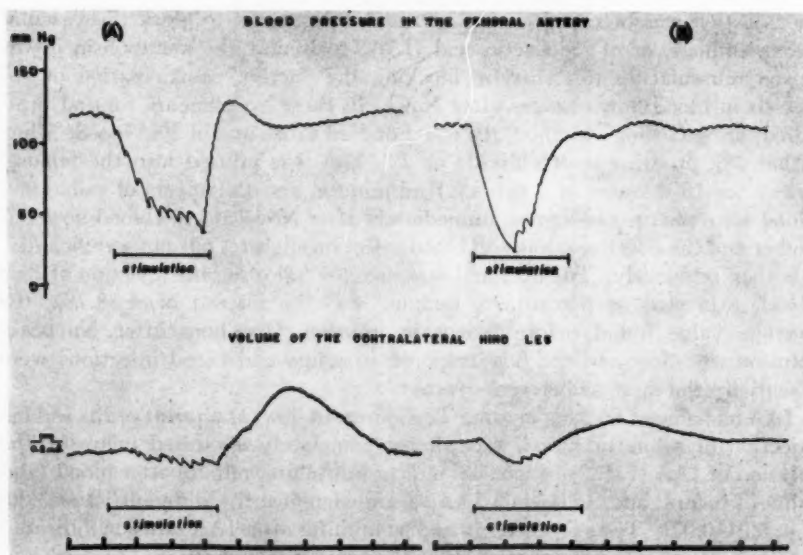


FIG. 3. Effect of the stimulation of the vagosympathetic trunk in dog on the simultaneous registered blood pressure and leg volume. In the experiment A the stimulus was of increasing (1→10 v) intensity, and in the experiment B the stimulus was of decreasing (10→1 v) intensity; in both cases for 15 seconds. B is 10 minutes later than A. Volume increase is more marked in experiment A, when the arterial pressure rise is more sudden than the fall. Time scale indicates 5-second intervals.

followed by a sudden rise, and in the second type a sudden fall was followed by a rather slow rise in pressure (Fig. 3). Volume increase in the dog's leg was longer lasting (60 seconds on the average) and was also more intensive in the first type of experiments than in the second type (where the average duration was only 38 seconds). While no relation was found between the abruptness of the arterial pressure fall and the subsequent volume increase, there tended to be a greater increase in the volume, when the rise in the arterial pressure was more rapid (Fig. 3).

4. Discussion

In our experiments a sudden injection of physiological saline into the femoral artery consistently increased blood flow at the site of the injection and distally from it. This increase of flow always lasted longer than the rise of pressure produced by the injection. The injection of Locke's solution or of blood had a similar effect, indicating that the decrease in vascular resistance cannot be explained by a decrease in the viscosity. Chambliss *et al.* (6) suggested that the increase in coronary flow caused by a rapid injection of blood or saline is due to ATP or some other materials released from the red cells. Such an explanation could hardly account for the fact that when the same injection is administered under identical conditions into either the femoral or the carotid artery, large differences in effect can be obtained.

Rapid injection of saline into the artery, if no vascular adjustment at all occurred, would increase the intravascular pressure, at least during the time of the injection. The arterial pressure, however, as we have seen, was increased only for a very short period, returned to about the control value even during the injection, and then went slightly below it. This would suggest that a vascular relaxation was the response to the stretching produced by the rapid injection. This vascular response, we feel, is an "overshooting" one, producing increased vessel-diameter for some time after the artery is stretched, i.e. even when the injection is over. We do not believe the nervous system to be important in this response. Indeed, vascular innervation was not intact in our experiments where Girling's method was used and the artery was doubly cannulated; furthermore, no significant difference was found in the effect of saline injections when Novocain (2%) was previously infused into the artery, in a concentration generally believed to block the afferent nerve impulses from the vessels.

Can the effect of sudden injections on the blood flow be explained entirely by the distension of the vessels, or would there be an "active" myogenic response involved? We feel that myogenic response in the sense as claimed by Bayliss (1) and by Folkow (2, 3), i.e. vasoconstriction resulting from sudden intravascular pressure increase, certainly did not occur in our experiments. Indeed, we found decreased vascular resistance in the first minute after the injections and increased resistance was not seen even in the next few minutes. Furthermore, the results of our experiments simulating those of Bayliss,

described in Section (g), indicate that the increase in the volume is the consequence of the sudden intra-arterial pressure increase rather than of the sudden drop in the blood pressure. It seems worth while to mention that Bayliss himself was apparently more reserved than others about his above-mentioned suggestions, for he considered the whole question as "undecided" in a later book (7). On the other hand, we feel that a myogenic factor can be involved, in another sense, in the vascular response to stretching. Blood flow was essentially less increased in our experiments in the carotid than in the femoral artery by the injection of saline or blood. The former artery contains less muscular tissue than the latter. These results can be compared with the *in vitro* experiments of Zatzman *et al.*, who found bigger stress relaxation in the umbilical artery which contains largely muscular elements than in the carotid, which contains little muscular tissue (8). Furthermore, our results after the administration of iodoacetic acid, which is reported to poison the vessel musculature in the concentration used in this study (5), add an argument for a myogenic factor in the flow alterations described in this paper. The "blocking effect" of IAA on the flow increase after saline or blood injection is unlikely to be explained simply by a previous maximal vasodilation caused by IAA. Blood flow after IAA, although increased, was still definitely lower than the maximal flow which we obtained with different vasodilators—acetylcholine, ATP, etc.—using the same method in a large number of dogs. Furthermore, in a few animals in this study, the increased flow after acetylcholine or Regitine still did not prevent the effect of saline injections. Consequently we feel that the "blocking effect" of IAA does not result from the production of a maximal vasodilation, which would abolish the possibility of further decrease in peripheral resistance after vascular stretching, but rather from the inhibition of an "active" response of the vessel muscles to the distension provoked by sudden injection of physiological saline or blood.

An alternative explanation of our experiments would be that the rapid injection of fluid caused the opening of vessels previously closed because their "critical closing pressure" (9) exceeded the actual intra-arterial pressure.

The conclusions from our experiments have also a "practical" aspect. They emphasize the importance of avoiding a sudden arterial injection, and of using instead a continuous infusion, where vasoactive drugs are being studied exactly.

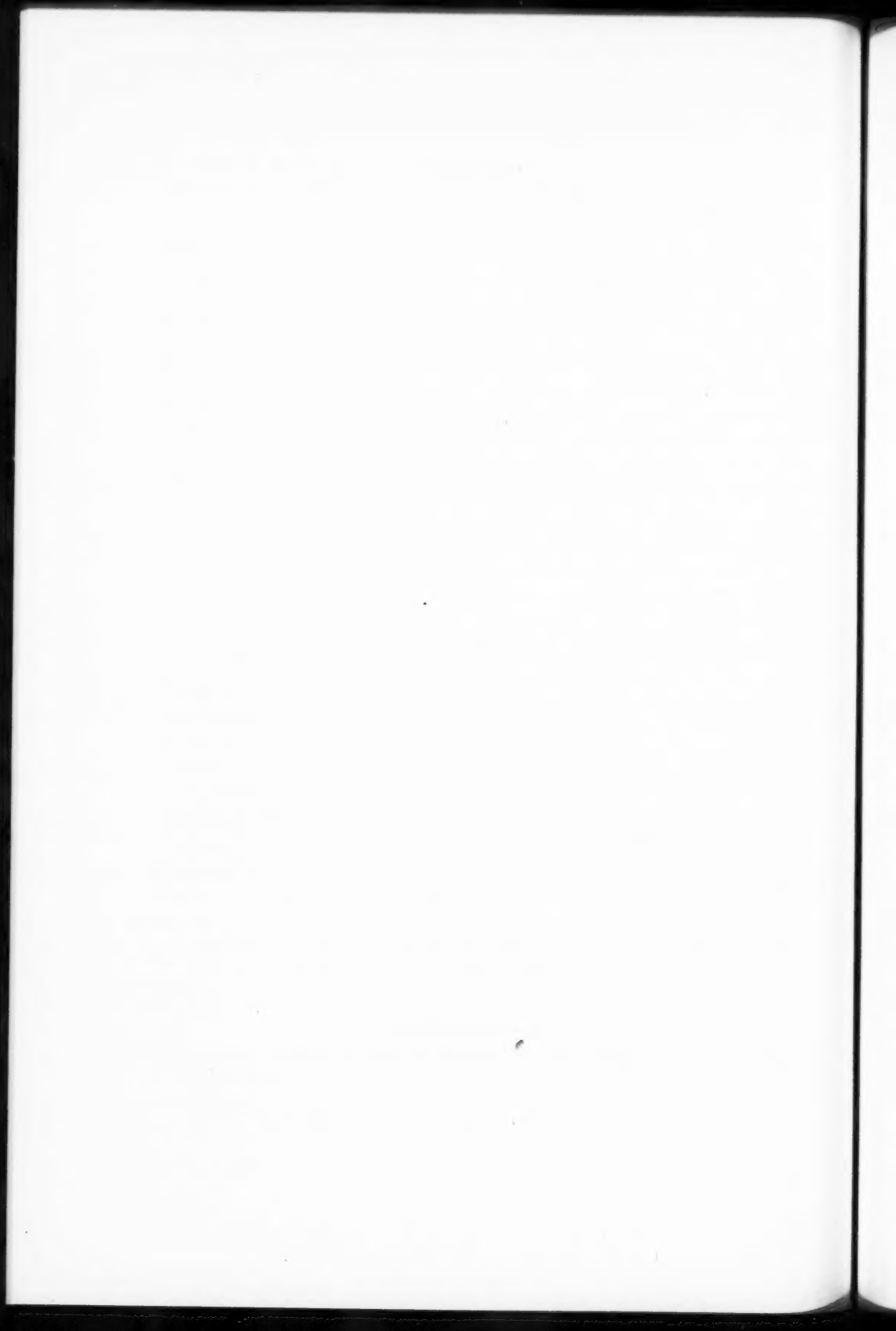
It would be of interest to compare the above-described alterations in blood flow following saline injections, in different vascular areas and also in diseased vessels.

Acknowledgment

The author is deeply grateful to Dr. A. C. Burton for his valuable criticisms and suggestions.

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ADENINE NUCLEOTIDE BREAKDOWN AND ITS RELATIONSHIP TO POLYNUCLEOTIDE PHOSPHORYLASE IN THE CROWN-GALL TUMOR INDUCING ORGANISM *AGROBACTERIUM TUMEFACIENS*¹

A. VARDANIS² AND R. M. HOCHSTER

Abstract

Cell-free extracts of the crown-gall tumor inducing organism *Agrobacterium tumefaciens* (strain B₆) have been shown to convert ATP to ADP relatively slowly. An adenylate kinase of only moderate activity has been found.

Crude extracts of this organism contain an extremely active polynucleotide phosphorylase which rapidly catalyzes the synthesis of polyadenylate from ADP, releasing P_i in stoichiometric proportions. Extracts are also shown to exhibit some phosphodiesterase activity towards the polyadenylate so formed.

Even though the enzymatic conversions of adenosine and of IMP to hypoxanthine are shown to occur readily it has not been possible to demonstrate any metabolic link between AMP and either adenosine or IMP. Extracts of *A. tumefaciens* have the unusual property of being unable to metabolize further AMP or other similar mononucleotides (with the exception of IMP).

It is shown also that polynucleotide phosphorylase is active not only in extracts of crown-gall tumor inducing organisms but also in those of at least one related non-tumorigenic species of *Agrobacterium*.

Introduction

The results to be reported in this paper are a part of a general study concerned with the relative importance of different metabolic pathways in *Agrobacterium tumefaciens*, the organism responsible for the crown-gall tumor disease of plants. It is felt that a thorough knowledge of the intermediary metabolism of the bacterium may facilitate a better understanding of the tumorigenic process by providing some clues on the nature of the tumor-inducing principle—the, as yet, hypothetical bacterial product believed to induce the transformation of normal plant cells to crown-gall tumor cells.

The only comprehensive study available in the literature of the pathways involved in the breakdown of ATP* by a single microorganism is that reported by Hochster and Madsen (1) for the phytopathogen *Xanthomonas phaseoli*. It is the purpose of this paper to present the results of a similar study with *Agrobacterium tumefaciens*.

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²National Research Council of Canada Fellow in collaboration with the Canada Department of Agriculture, 1959–1961.

*The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate (adenylic acid); IMP, inosine monophosphate (inosinic acid); CMP, cytidine monophosphate (cytidylic acid); UMP, uridine monophosphate (uridylic acid); GMP, guanosine monophosphate (guanylic acid); GSH, glutathione; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; P_i, inorganic phosphate.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: ATP, ADP, AMP, adenosine, adenine, IMP, inosine, hypoxanthine, and 2',3'-AMP mixed isomers (Pabst Brewing Co.); GSH (Schwarz Laboratories) and Tris (Sigma Chemical Co.).

Agrobacterium tumefaciens (strains A₆ and B₆) was obtained from Dr. A. C. Braun, Rockefeller Institute for Medical Research, New York; *Agrobacterium radiobacter* (No. 590) from our own Culture Collection; *Agrobacterium radiobacter* (strain R-1001) from Dr. E. B. Roslycky, Pesticide Research Institute, Canada Department of Agriculture, London, Ontario; *Agrobacterium rubi* (P₆₀) from Dr. H. Katznelson of the Microbiology Research Institute, Canada Department of Agriculture; and *Agrobacterium rhizogenes* from Dr. P. A. Ark, Department of Plant Pathology, University of California, Berkeley.

Enzyme Preparations

All the organisms used were grown in a medium consisting of 1% yeast extract and 1% glucose in Fernbach flasks on a shaker for 18 hours. The cells were then harvested, washed, and treated in the 10-kc Raytheon oscillator as previously described (1). The "whole extract" thus obtained was dialyzed overnight against distilled water at 0–3° C, before use. All results given in this paper, with the exception of those included in Table I, were obtained with *Agrobacterium tumefaciens*, strain B₆.

Incubation

Incubation temperature was 30° C in all experiments. Reactions were stopped by the addition of TCA to a final concentration of 5%. The mixtures were chilled, centrifuged, and aliquots of the supernatant solutions were used for analysis. For chromatography the supernatants were extracted three times with two volumes of diethyl ether to remove the TCA.

Analytical Methods

The following solvent systems were used in descending chromatography for the isolation of compounds of interest in this study:

Solvent A: Isobutyric acid – concentrated ammonia – water (66:1:33). R_f values obtained: ATP, 0.23; ADP, 0.36; AMP, 0.53; inosine, 0.50; hypoxanthine, 0.64; adenosine, 0.81.

Solvent B: Saturated ammonium sulphate – *M* sodium acetate – isopropanol – 1% versene (80:18:2:11). R_{S-AMP} values* obtained: 3'-AMP, 1.0; 2'-AMP, 1.51; ADP, 2.27.

Solvent C: Ethyl acetate – acetic acid – water (3:3:1) (2). R_f values obtained: ribose, 0.49; xylose, 0.42; arabinose, 0.40.

In the studies on the breakdown of nucleotides, aliquots of the supernatants were spotted on sheets of Whatman No. 1 filter paper alongside known reference

* R_{S-AMP} refers to values obtained for the movement on filter paper sheets of the substances indicated relative to the movement of 3'-AMP under the same experimental conditions.

compounds and chromatographed for 18 hours in solvent system A. The compounds were located according to their absorption when exposed to a Minera-light (Model SL 2537, with short-wave ultraviolet filter). Where clear-cut identification was difficult (as with AMP and inosine) the respective ultraviolet-absorbing areas were cut out, eluted, and their spectra traced with a Beckman DK-1 recording spectrophotometer. For the quantitative estimation of these compounds, 0.1 ml of the supernatant solutions from deproteinized incubation mixtures was streaked on paper, chromatographed, and the substances located in the same manner with parallel known markers. The individual spots were eluted with a small quantity of distilled water and diluted to an appropriate volume with 0.05 *M* tris buffer (pH 7.0). The buffered solutions were then analyzed in a Beckman DU spectrophotometer. A molar extinction coefficient of 15,400 at 259 *mμ* was used to calculate the concentrations of the adenine compounds and 12,200 at 249 *mμ* for the inosine compounds.* For the identification and quantitative estimation of ribose, 0.1 ml of the supernatant solution was streaked on sheets of Whatman No. 1 filter paper and developed for 18 hours in solvent system C together with known markers of ribose, xylose, and arabinose. The sugars were located following reaction with the *o*-amino-biphenyl spray (3). After positive identification of the product as ribose, the remaining unsprayed strips of paper were eluted with distilled water and the eluates were subjected to the Mejbaum test (4).

Inorganic phosphate and total phosphate were determined by the method of Fiske and SubbaRow (5). The protein concentration was estimated routinely by the spectrophotometric method of Warburg and Christian (6). For the experiments reported in Table I the more precise method of Lowry *et al.* (7) was used.

Isolation, Purification, and Characterization of the Polyadenylate

ADP (500 mg) was incubated with 10 ml of whole extract in a total volume of 30 ml; the reaction mixture also contained 500 *μ*moles of tris buffer (pH 8.1) and 100 *μ*moles of MgCl₂. After 60 minutes' incubation the reaction mixture was precipitated with two volumes of absolute ethanol at 0–3° C and was left to stand for a further 60 minutes at this temperature. The precipitate was spun down at 12,000 r.p.m. for 10 minutes and the supernatant was discarded. The sediment was taken up in a minimum quantity of distilled water and the precipitation procedure was repeated. The final precipitate was again taken up in distilled water and the extremely viscous solution was clarified by centrifugation at 15,000 r.p.m. for 5 hours in a Spinco Model L centrifuge. The clear supernatant was again treated with two volumes of ethanol, and the precipitate was taken up in distilled water. An aliquot of this solution was then hydrolyzed in 1 *N* NaOH for 48 hours at room temperature. After this time, TCA was added to the solution until a pH of 2 was reached. A small amount of protein which had precipitated at that stage was removed by centrifugation at 10,000

*Extinction values at the pH used in this study were those published by Pabst Laboratories, Milwaukee, Wisconsin, Circular No. OR-15.

r.p.m. for 15 minutes. The preparation was then extracted three times with two volumes of diethyl ether to remove the TCA. An aliquot of the final solution was spotted on Whatman No. 1 filter paper and developed in solvent system B for 20 hours together with suitable known reference substances.

Viscometry

The rate of polynucleotide phosphorylase activity was measured as a function of viscosity increase of the incubation medium with time. A standard capillary viscometer was employed. In calculating the results the following equation was used (8):

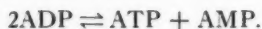
$$\frac{\eta}{\eta_0} = \left(\frac{t}{t_0} \right) \left(\frac{\rho}{\rho_0} \right)$$

where η , t , and ρ are the viscosity, outflow time, and density, respectively, for the experimental system (with added ADP) and η_0 , t_0 , and ρ_0 are the same units for the control system (without added ADP). Since ρ and ρ_0 were not likely to change during the incubation the expression (ρ/ρ_0) would be expected to remain constant for all determinations and was therefore omitted. The final results were expressed as specific viscosity (i.e. $\{(\eta/\eta_0) - 1\}$).

Experimental and Results

The Metabolism of ATP, ADP, and AMP

An extract of the organism was incubated with ATP in order to obtain a time course of the degradation of this substance and to obtain quantitative evidence for the appearance of the breakdown products. The results are given in Fig. 1B. It can be seen readily that the disappearance of ATP and the formation of ADP were found to be relatively slow processes. The next step was an attempt to elucidate the fate of ADP when it was supplied as a substrate to an extract of *A. tumefaciens*. The results obtained are presented in Fig. 2. They lead to a number of considerations. First, it was evident that some adenylate kinase activity was present in our extract. During the first 20 minutes of incubation, approximately equal amounts of ATP and of AMP appeared in the incubation mixture suggesting the adenylate kinase reaction



However, the absolute amounts of the products of the adenylate kinase reaction could not at any time account for the number of micromoles of ADP that had disappeared. In the course of the chromatographic isolation of these compounds no other ultraviolet-absorbing products (other than ATP, ADP, and AMP) could be detected. Measurements of inorganic phosphate in these reaction mixtures revealed a release of P_i equimolar to the amount of ADP that had disappeared and which could not be accounted for on the basis of the adenylate kinase reaction alone. Furthermore, during the course of these experiments there was a clearly visible increase in the viscosity of the incubation

TABLE I
Polynucleotide phosphorylase activity as measured by increase in viscosity

Incubation time (minutes)	Specific viscosity $\{(\eta/\eta_0) - 1\}^*$							
	Experiment I			Experiment II				
	<i>A. tumefaciens</i> (B ₀)†	<i>A. radiobacter</i> (590)	<i>A. rubi</i> (P ₀)	<i>A. tumefaciens</i> (A ₀)†	<i>A. tumefaciens</i> (B ₀)†	<i>A. radiobacter</i> (R-1001)	<i>A. rubi</i> (P ₀)	<i>A. rhizogenes</i>
0	0	0	0	0	0	0	0	0
5	0.77	0.41	0.04	0.26	0.49	0.32	0.09	0.13
10	2.04	1.09	0.07	0.82	1.30	0.98	0.13	0.19
15	3.20	1.80	0.11	1.50	2.09	1.72	0.18	0.22
20	4.06	2.46	0.12	2.26	2.85	2.48	0.21	0.24
25	4.61	3.06	0.15	2.95	3.64	3.18	0.26	0.26
30	4.84	3.57	0.17	3.67	4.42	3.82	0.30	0.26
35	4.84	3.98	0.20	—	—	—	—	—
45	4.36	4.41	0.25	5.08	6.01	5.03	0.37	0.26
60	3.26	4.44	0.28	—	—	—	—	—

NOTE: Contents of reaction mixture: ADP (where used), 100 μ moles; MgCl₂, 20 μ moles; tris buffer (pH 8.1), 100 μ moles; whole extract containing 35 mg protein. Total volume: 6.0 ml.

*Where η_0 = viscosity (in poises) of the control, η = viscosity (in poises) of the experimental with added ADP; no density correction has been applied. For derivation of this relationship, see Schachman (8) and the explanation in Materials and Methods.

†Crown-gall-inducing organisms.

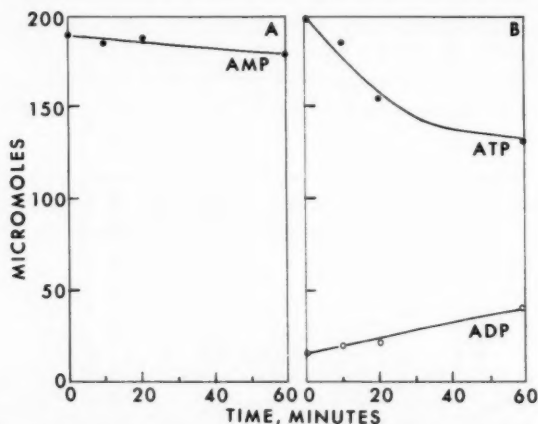


FIG. 1. The breakdown of AMP (A) and of ATP (B) by an extract of *A. tumefaciens*. Contents of reaction mixture: AMP or ATP, 200 μ moles; NaHCO_3 , 120 μ moles; MgCl_2 , 20 μ moles; whole extract, 2 ml. Total volume: 6.0 ml.

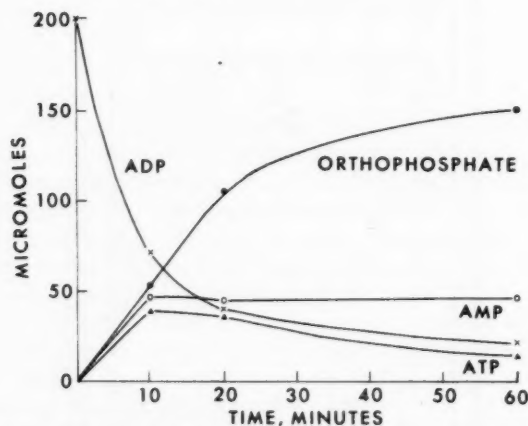
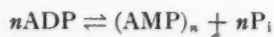


FIG. 2. The breakdown of ADP by an extract of *A. tumefaciens*. Contents of reaction mixture: ADP, 200 μ moles; NaHCO_3 , 120 μ moles; MgCl_2 , 20 μ moles; whole extract, 2.0 ml. Total volume: 6.0 ml.

mixture. The above results could best be explained if it could be shown that ADP was undergoing the reaction



representing the synthesis of polyadenylate and being catalyzed by polynucleotide phosphorylase, the enzyme discovered and described for a number of microorganisms by Ochoa and his co-workers (9, 10, 11). This reaction would explain our observations of: (a) the increase in viscosity in the incubation

mixture, (b) the release of amounts of P_i equimolar to the disappearing ADP, and (c) the net disappearance of ultraviolet-absorbing material, as the polynucleotide would be precipitated in the routine deproteinization of the samples with TCA (see Materials and Methods).

Subsequent experiments proved the above prediction to be correct. The polynucleotide isolated from ADP-incubation experiments in the presence of the extract was purified and subjected to alkaline hydrolysis (see "Materials and Methods"). On chromatography, the hydrolyzate was found to contain two ultraviolet-absorbing spots, corresponding to the known 2' and 3' isomers of AMP (Fig. 3). This was the expected isomeric mixture from alkali-treated

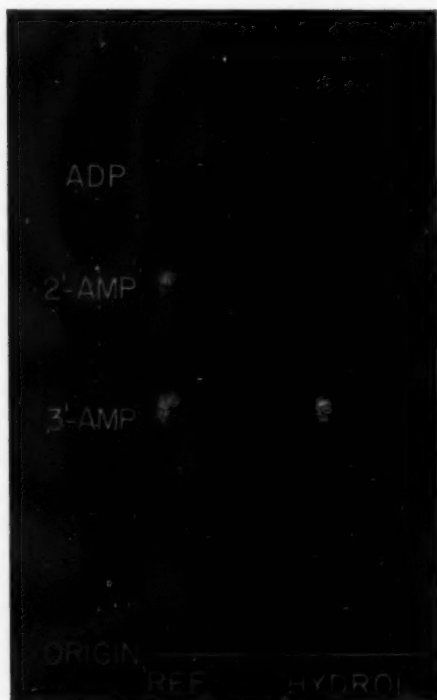


FIG. 3. Chromatography (solvent system B) of the products of alkaline hydrolysis of the polynucleotide synthesized from ADP by extracts of *A. tumefaciens*.

AMP polynucleotide chains made up of nucleoside residues linked by 3',5' phosphodiester bridges (11). The monophosphate nature of the products of hydrolysis was confirmed by analysis for total phosphorus. An aliquot of the hydrolyzate containing 1.50 μ moles of adenine (calculated from its optical density at 260 $m\mu$) was found to contain 1.51 μ moles of phosphorus (5).

The results to date show that the polynucleotide phosphorylase of *A. tumefaciens* is an extremely active enzyme even in crude extracts. This finding suggested a possible role of this enzyme system in tumorigenesis. To test this possibility, experiments were set up in which the activities of the polynucleotide phosphorylases of tumorigenic and of non-tumorigenic species of *Agrobacteria* were tested side by side. In all cases ADP was used as the substrate and increase in viscosity was used as the measure of activity (see Materials and Methods). Table I summarizes the results of these experiments. It is clear that the crown-gall inducing organism (strains A₆ and B₆) shows considerable polynucleotide phosphorylase activity which is, however, shared by two strains of one non-tumorigenic organism (strains 590 and R-1001). Very little activity was noted in extracts of *A. rubi* and of *A. rhizogenes*. The results indicate that rapid synthesis of a polyadenylate is probably not a specific function of only the tumorigenic species of *Agrobacterium*.

Experiments I and II (Table I) were carried out with two separate extracts obtained by growth of the cells in media in which yeast extract and glucose were autoclaved together (experiment I) or separately (experiment II). It was observed in experiments with *A. tumefaciens* (B₆), carried on for longer periods of time than are shown in Table I, that the specific viscosity would increase as shown, reach a maximum (varying from 30 to 90 minutes from one extract to another), and then return to zero, usually in 8 to 10 hours. Several such reaction mixtures were treated with TCA to a final concentration of 5% after the specific viscosity had again returned to zero. Aliquots of the supernatant solutions were subjected to paper chromatography followed by spectrophotometric analysis, and were found to contain mainly 5'-AMP and traces of ADP. The sum of these two components (as calculated from the optical density of the supernatant solution at 259 m μ) accounted completely for the amount of ADP initially added to the mixture. The above results are taken to suggest the presence of an enzyme in extracts of *A. tumefaciens* (B₆) similar to snake venom phosphodiesterase which hydrolyzes 3'-5' internucleotide links in such a way as to leave phosphate esterified in the 5' position (11). Thus it is possible that differences in individual extracts with respect to the attainment of maximum specific viscosity (and its subsequent decrease) are due to a variation from extract to extract in the relative enzymatic activities of polynucleotide phosphorylase and of phosphodiesterase.

A subsequent study concerned with the degradation of AMP showed that added AMP could be almost quantitatively recovered at any time during incubation with the extract (see Fig. 1A). Incubation of other nucleotide monophosphates (CMP, UMP, GMP) with cell-free extracts of *A. tumefaciens* led to very similar results. CMP and UMP did not disappear at all while GMP gave rise to a trace of guanosine.* These results are unique when compared with other bacterial and animal tissues in which nucleotide monophosphate degradation takes place readily.

*Experiments carried out in our laboratory by Mrs. C. G. Nozzolillo.

The above results were confirmed by an independent manometric study in which the extracts were incubated separately with ATP, ADP, AMP, IMP, adenosine, and adenine in a bicarbonate medium (1). The results obtained are shown in Fig. 4. Incubation of extracts (without added substrate) with ATP

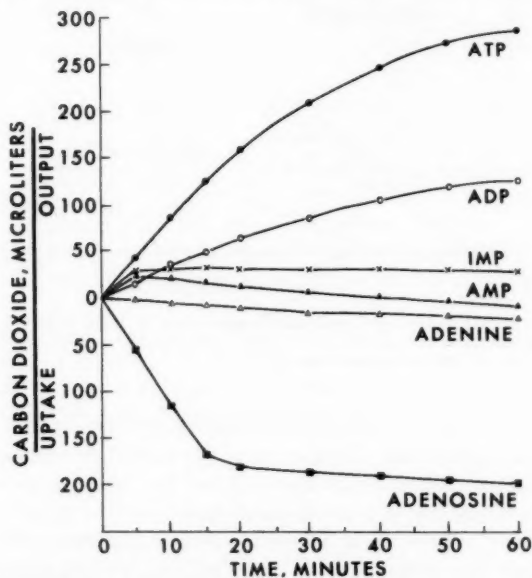


FIG. 4. Manometric determinations of phosphorylation (12) and of deamination reactions (1) (net values). Vessel contents: NaHCO_3 , 90 μmoles ; MgCl_2 , 10 μmoles ; whole extract, 1 ml; ATP, ADP, AMP, IMP, adenosine or adenine, 15 μmoles . Gas phase: 95% N_2 + 5% CO_2 ; total volume: 3 ml.

resulted in some CO_2 output (12). This could be due to endogenous substrate phosphorylation or perhaps to some ATP-ase activity. The CO_2 output obtained when ADP was used as substrate was probably the result of ATP formation by the action of adenylate kinase. It is not likely that dephosphorylation of ADP took place since equimolar amounts of AMP and of ATP were formed when extracts were incubated with ADP (see Fig. 2). It was shown by Hochster and Madsen (1) that enzymatic deamination carried out under these experimental conditions would manifest itself as a CO_2 uptake. It is evident, from Fig. 4, that in keeping with previous results with regard to the lack of AMP disappearance, the addition of this compound did not cause any gas uptake or output. On the other hand the CO_2 uptake resulting from the addition of adenosine suggested the presence of adenosine deaminase.

The Metabolism of Adenosine and of IMP

The chromatographic isolation, identification, and analytical measurement of the products of adenosine degradation showed (Fig. 5) that there was a rapid

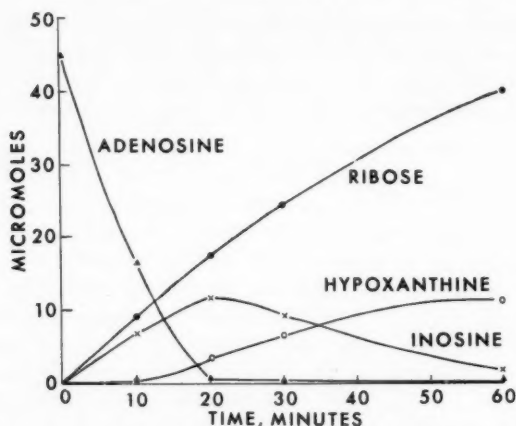


FIG. 5. The breakdown of adenosine by an extract of *A. tumefaciens*. Contents of reaction mixture: adenosine, 45 μ moles; tris buffer (pH 7.0), 100 μ moles; $MgCl_2$, 20 μ moles; whole extract, 2.0 ml. Total volume: 6.0 ml.

disappearance of adenosine followed by a concomitant formation of inosine and of hypoxanthine respectively. At the end of the period of incubation, however, it was not possible to account quantitatively for the disappearance of adenosine on the basis of the number of micromoles of inosine and of hypoxanthine found. When the amount of free ribose in the incubation medium was measured an unexpected result was obtained (Fig. 5). The rate of formation of ribose, the by-product of the inosine to hypoxanthine reaction (1), would be expected to follow the hypoxanthine curve. Instead, it appeared at a much earlier stage and accumulated in the medium to a concentration of approximately four times that of hypoxanthine. On the other hand, the sum of ribose and inosine could now account for the amount of adenosine that had disappeared after 60 minutes of incubation. One possibility that could explain these findings would be the further degradation of hypoxanthine at a rate slightly lower than that of its formation. This possibility must be discounted, however, as subsequent experiments proved that hypoxanthine could be recovered quantitatively even after 60 minutes' incubation with the extract. Thus, although the degradation of adenosine to inosine and to hypoxanthine has been demonstrated the quantitative aspects of this pathway are not clear as yet. The possibility of the occurrence of other side reactions unknown at present cannot be ruled out but must await further study.

It was then considered necessary to decide whether a 5'-nucleotidase acting on IMP was present in these extracts. Incubation of extracts with IMP resulted in a gradual release of inorganic phosphate (Table II), indicating the presence of this enzyme. Chromatography of samples taken at different times during this incubation, and subsequent spectrophotometric analysis of the

TABLE II
Dephosphorylation of IMP

Incubation time (minutes)	μ moles inorganic phosphate released (net)
0	0.6
5	10.3
10	18.7
15	22.6
30	31.0
45	37.4
60	43.2

NOTE: Contents of reaction mixture: IMP, 50 μ moles; $MgCl_2$, 20 μ moles; tris buffer (pH 7.0), 100 μ moles; whole extract, 2.0 ml. Total volume: 6.0 ml.

three ultraviolet-absorbing spots obtained, showed the disappearance of IMP and the successive appearance of inosine and of hypoxanthine. Quantitative estimation of the starting material and of its two products was not undertaken.

Discussion

The scheme in Fig. 6 summarizes the metabolic pathways identified in the metabolism of adenine nucleotides by extracts of *A. tumefaciens*. Reaction 1, the hydrolysis of the terminal phosphate grouping of ATP, has been shown by

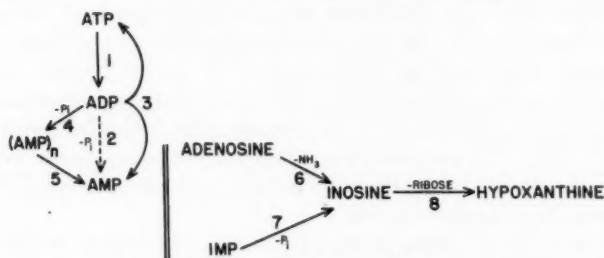


FIG. 6. Summary of the pathways concerned with adenine nucleotide metabolism in extracts of *A. tumefaciens*.

Hochster and Madsen (1) to be an extremely active process in extracts of another phytopathogenic organism (*Xanthomonas phaseoli*). With *A. tumefaciens*, the disappearance of ATP and the formation of ADP is comparatively slow (Fig. 1B). The shape of the ATP curve in Fig. 1B, as well as the CO_2 evolution obtained by the addition of ATP in the absence of any added substrate (Fig. 4), indicates that ATP dephosphorylation can be attributed, at least in part, to reactions concerned with the phosphorylation of endogenous substrates. Reaction 2, the direct dephosphorylation of ADP, does not seem to play a very significant role in extracts of *A. tumefaciens*. The quantity of AMP formed from ADP was accompanied by the formation of approximately equimolar amounts of ATP (Fig. 2), indicating that reaction 3 was the only one operating. Adenylate kinase activity (reaction 3) was previously found to

be extremely high in extracts of *X. phaseoli* (1). By comparison, extracts of *A. tumefaciens* exhibited only moderate activity. This could be due to the relative lack of availability of ADP because of the rapid removal of the latter from the incubation mixture by the extremely active polynucleotide phosphorylase (reaction 4).

The striking feature about the polynucleotide phosphorylase of *A. tumefaciens* is its high activity in crude extracts and its probable dominance in competing systems in which ADP is formed as an intermediate or is required as a phosphate acceptor. Previous failures to obtain oxidative phosphorylation in studies with this organism (13) can now be explained, at least in part, by the presumed unavailability of ADP as a phosphate acceptor despite the fact that considerable quantities of ATP were used.

Reaction 5, catalyzed by a hydrolytic enzyme similar to venom phosphodiesterase (11), is extremely slow and its role in the over-all metabolic picture cannot as yet be assessed with certainty.

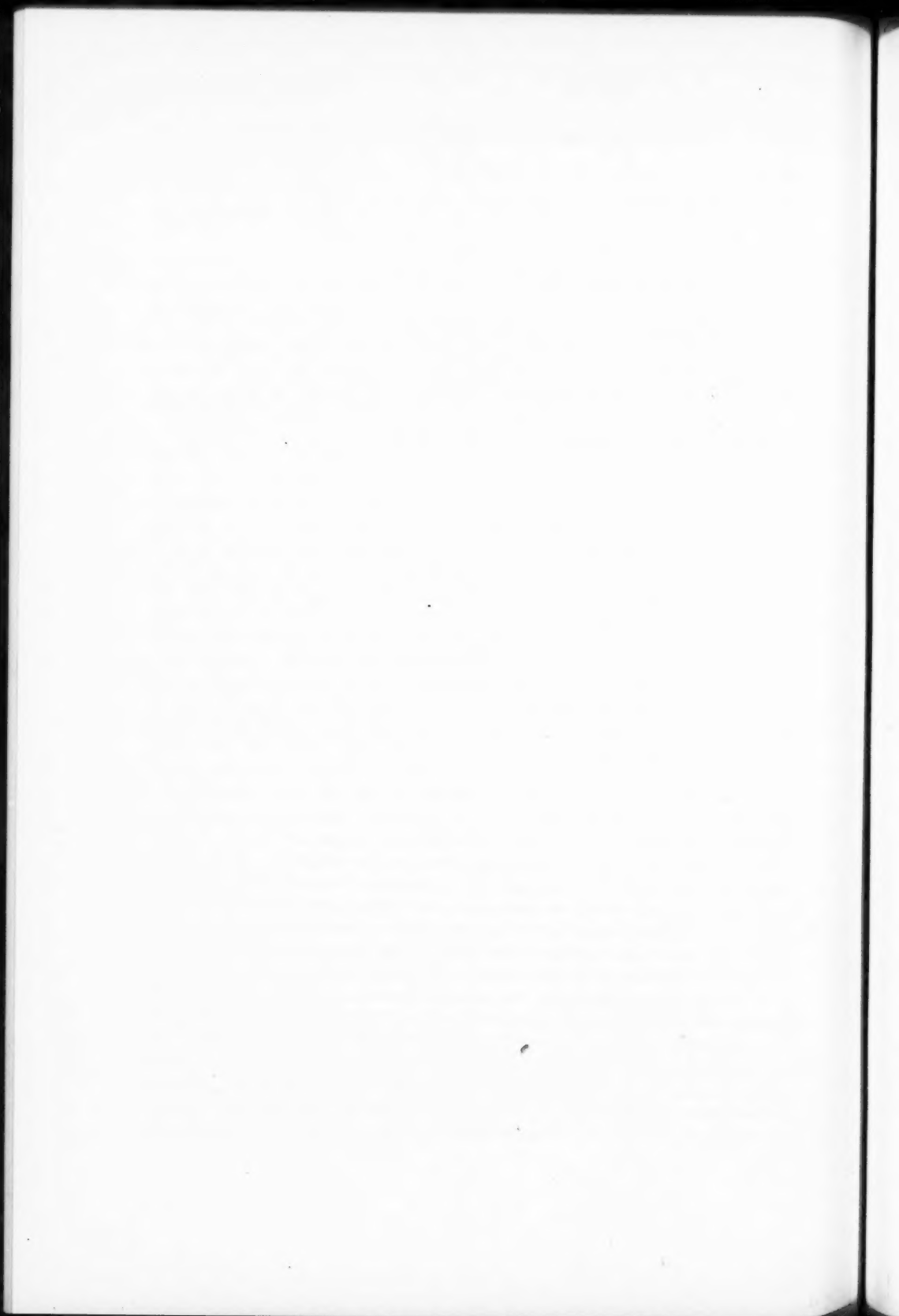
Another interesting observation arising from the present study is the fact that we were unable to establish a metabolic connection between AMP and IMP or between AMP and adenosine. This contrasts sharply with the results which Smillie (14) obtained with brain tissue and others from this laboratory (1) with the phytopathogenic organism *X. phaseoli*. Smillie observed the degradation of AMP via IMP and to some extent also via adenosine. The studies in extracts of *X. phaseoli* showed a specific degradation route from AMP through IMP to hypoxanthine (1). The work reported in this paper shows that, in extracts of the crown-gall tumor inducing organism, reactions 6, 7, and 8 occur but that there is no demonstrable direct link with the metabolism of the adenosine phosphates (see Fig. 6). This observation was extended further by the finding that other nucleotide monophosphates (CMP, UMP, GMP) behave in a similar manner.

By analogy with the detailed studies on the metabolism of the adenosine phosphates it would seem that nucleotide monophosphates (with the exception of IMP) are not themselves metabolized in extracts of *A. tumefaciens*. Instead, their related nucleotide diphosphates may preferentially enter into the polynucleotide phosphorylase reaction in this organism. The fact that the monophosphates are not metabolized further supports the thermodynamic feasibility of the ready conversion of the diphosphates to their respective polynucleotides.

Whereas the presence of such a highly active polynucleotide phosphorylase does not in itself appear to provide a direct clue to a possible tumorigenic function, in view of its presence also in one non-tumorigenic species, it is tempting to speculate that polynucleotides formed by the action of this enzyme may assume the role of soluble RNA in certain *Agrobacteria*. Specificity with respect to tumorigenesis could then be a function of the arrangement of the particular bases in the soluble RNA of the tumorigenic vs. the non-tumorigenic species. Further work is now being pursued in our laboratory designed to provide an answer to this most intriguing question.

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ACETYLCHOLINE METABOLISM OF A SYMPATHETIC GANGLION¹

R. BIRKS AND F. C. MACINTOSH

Abstract

The synthesis, storage, and release of acetylcholine (ACh) were studied in perfused and intact superior cervical ganglia of cats. ACh was determined by bio-assay in ganglion extracts and in the venous effluent from ganglia perfused with fluid containing an anticholinesterase drug. Of the extractable ACh in a normal ganglion, about 85% is "depot ACh" available for release by nerve impulses. This must be located in the nerve endings; most of the remainder is in the intraganglionic portions of the preganglionic axons. The depot ACh exists as two fractions, of which one, the smaller, is the more readily available for release by nerve impulses. ACh synthesis and release go on at a measurable low rate in the absence of nerve impulses; both are greatly accelerated by activity. Under physiological conditions of excitation and perfusion, ACh release does not outrun ACh synthesis; but synthesis is slowed, with consequent depletion of depot ACh and reduction in ACh release, if choline is absent from the extracellular fluid, or if the hemicholinium base HC-3 is present. The latter compound specifically inhibits ACh synthesis by competing with choline; as a result it produces delayed block in repetitively activated cholinergic pathways. For efficient synthesis of ACh during experiments lasting an hour or so, a ganglion need be supplied with no substances other than choline and the constituents of Locke's solution; for the efficient release of ACh, the perfusion fluid must also contain CO₂ and an unidentified factor present in plasma. ACh accumulates above the resting level in a ganglion whose cholinesterase has been inactivated, provided that the perfusing fluid is one that supports ACh synthesis; the additional ACh is not immediately available for release by nerve impulses. Under physiological conditions of perfusion the amount of ACh set free by each maximal preganglionic volley is highest in a ganglion that has been at rest, and is then independent of stimulation frequency; after repetitive activation for several minutes the volley output is lower and is only frequency-independent at rates of excitation below 20/second. Consideration is given to the probable intracellular locations of the several fractions of the ganglionic ACh, and to their interrelationship.

Introduction

The metabolism of acetylcholine (ACh) at active nerve endings was first studied quantitatively by Brown and Feldberg (1). In their procedure the superior cervical ganglion of a cat was perfused with eserinizied Locke's solution and subjected to prolonged repetitive stimulation by way of its preganglionic trunk; the ACh released from the activated nerve endings diffused into the perfusion fluid, and could be estimated in successive samples of the venous effluent; and finally the ganglion and its unstimulated fellow were extracted with trichloroacetic acid and the ACh content of each was determined. In such experiments Brown and Feldberg found regularly that the rate of ACh liberation was high at the beginning of stimulation, but fell off progressively to reach, after 20-30 minutes, a much lower level, which was then maintained with little further decline. It might have been expected that the ganglion would be found at this point to have lost most of its initial store of ACh, especially as the total

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Contribution from the Department of Physiology, McGill University, Montreal, Que.

amount it had released exceeded the amount it had contained to start with. In fact no such loss was found: the stimulated ganglion and the control, when extracted, yielded about the same amount of ACh. Thus, although degeneration experiments (2, 3) showed clearly that most of the extractable ACh of the ganglion must be in the nerve endings, it remained in doubt how far this ACh could be taken to represent the depot from which the transmitter is discharged when nerve impulses arrive at the synapses.

Other workers have confirmed the findings of Brown and Feldberg, and on the basis of their own experiments have tried to explain the paradoxical association of a declining ACh output with a well-maintained store of ganglionic ACh. Thus Kahlson and MacIntosh (4) suggested that stimulation did deplete the ACh depots, but that the deficit went unobserved because ACh was rapidly reformed in the short interval between removal of the ganglion and its disintegration in the extracting medium. Perry (5) advanced the hypothesis that only part of the extractable ACh of a ganglion is readily available for release by nerve impulses, and that while the ACh released by stimulation (under the usual conditions of perfusion with eserinated Locke's solution) is quickly replenished by synthesis, the newly formed ACh only becomes 'available' at a relatively slow rate, which is equal to the steady rate of ACh output after prolonged stimulation.

In the present study we have again measured, with some improvements in technique, the ACh output and content of ganglia perfused with eserinated Locke's solution, and we have obtained results that help to account for those of Brown and Feldberg. We have also done parallel experiments with perfusion fluids of altered composition, and have found that the synthesis and the release of ACh are to some degree independent processes for which the rate-controlling factors are different. Our results have enabled us to identify several of these factors, and appear to throw light on some of the processes involved in the turnover of ACh at nerve endings.

Some of our findings have already been presented briefly (6, 7, 8).

Methods

The experiments were carried out on cats. Medium-sized healthy animals of either sex were chosen. Anaesthesia was induced with ethyl chloride followed by ether, and maintained with chloralose.

Perfusion

The superior cervical ganglion, usually on the right side, was prepared for perfusion essentially by the method of Kibjakow (9) as modified by Feldberg and Gaddum (10). The main postganglionic trunk was left intact in order that the response of the nictitating membrane could be recorded. Particular care was taken to separate this trunk from accompanying arterial twigs through which reflux of blood into the perfusion stream might occur: with this precaution the perfusion pressure could nearly always be kept well below 100 mm Hg.

Since oedema and leakage were thus minimized the pressure-flow characteristics of the preparation usually changed little during an hour or more of perfusion, and little adjustment of the pressure was needed to keep the flow within the range 0.2–0.4 ml/minute. The perfusion fluid had previously been filtered through sintered glass and equilibrated with O_2 or a CO_2 – O_2 mixture. It was placed in a separatory-funnel reservoir, which was connected to a tank containing the same gas at the desired perfusion pressure, and was led to the ganglion by fine-bore polyethylene tubing, in which it was warmed to body temperature ($38 \pm 1^\circ C$) in the way described by Emmelin and MacIntosh (11). The arterial cannula had a capacity of less than 0.1 ml and was closed by a rubber stopper; the tubing from the reservoir was fitted to the ground-down butt of a fine hypodermic needle whose point was thrust through the stopper. When more than one perfusion fluid was to be used in an experiment, each was put into a similar reservoir and was led to the cannula in the same way. The perfusion could then be switched over to a fluid of different composition without losing time or disturbing the preparation, merely by turning the stopcocks of the appropriate reservoirs. Samples of venous effluent were collected in chilled tubes and stored at 0° until they were assayed not more than 3 hours later.

Perfusion Fluids

Locke's solution was freshly prepared and in most of the experiments had practically the same composition as that used by Brown and Feldberg (1) and by Perry (5); in g/l., NaCl 9.0, $KHCO_3$ 0.56, $CaCl_2$ 0.24, glucose 1.0, eserine sulphate 0.005 ($8 \times 10^{-6} M$). It was usually oxygenated at room temperature, and its pH was then about 8.5. In a few experiments in which it was equilibrated (at 20°) with 3.5% CO_2 in O_2 , the bicarbonate was raised to $2.5 \times 10^{-2} M$ by addition of $NaHCO_3$ and the NaCl was reduced correspondingly: the pH after treatment with CO_2 was then 7.4 ± 0.1 (at 38°).

Plasma was obtained on the day of the experiment by arterial bleeding of lightly etherized cats and was made incoagulable with heparin sodium (Connaught, 0.5 mg/ml). Plasma from human donors was obtained by venipuncture, the blood being drawn under negative pressure into bottles containing heparin, with sterile precautions. In either case eserine sulphate (2×10^{-5} g/ml; $3 \times 10^{-5} M$) was added, and the plasma was gassed with O_2 or CO_2 – O_2 in a large tonometer.

Plasma dialyzates were prepared by dialyzing 50-ml quantities of plasma overnight at 4° , with stirring, against 500 ml of distilled water. They were lyophilized and reconstituted to give an isotonic solution for perfusion.

HC-3 (hemicholinium compound No. 3 (12)), as the dibromide, was generously supplied by Dr. F. W. Schueler. Its concentration when added to Locke or plasma was $2 \times 10^{-5} M$. Tetraethyl pyrophosphate (TEPP) solutions were made up by dilution of sealed stock of the pure fluid (Eli Lilly & Co., Albright & Wilson Ltd.) immediately before use. A stock solution of eserine sulphate (Merck, $3 \times 10^{-3} M$) was made in saline and kept in the cold for not more than

a week; this was added in the required volume to the perfusion fluid just before each experiment. The molecular weight of the salt was taken as 648.5.

Stimulation

Stimulation of the preganglionic trunk was always begun 15 minutes after the start of the perfusion. The stimuli were rectangular voltage pulses of 1.0 millisecond duration and appropriate frequency, delivered through agar-saline pore electrodes via chlorided silver wires. The electrodes were transformer-coupled to the stimulator and the animal was grounded. During periods of prolonged high-frequency stimulation the electrodes were moved along the nerve towards the ganglion every 5 minutes: in spite of this precaution the stimuli sometimes fell temporarily below maximal, as was indicated by the nictitating-membrane record. Random irregularities of the ACh output curve could usually be attributed to this cause; but this source of error could not have been serious when conclusions were based on the pooled data from several experiments. It should be noted that the nictitating-membrane record in experiments of this kind is not always a reliable guide to the effectiveness of stimulation. This was particularly true in ganglia whose cholinesterase was inactivated and whose ACh output was high: under these conditions, repetitive stimulation floods the ganglion with free ACh (1); synaptic transmission is partly or wholly blocked although the ganglion cells may be firing rapidly and asynchronously (13); as an after-effect of their prolonged depolarization, asynchronous firing may persist for a long time after stimulation is over (cf. Emmelin and MacIntosh (11)).

Ganglion Extracts

It has been shown that cold trichloroacetic acid efficiently extracts ACh from nervous tissue (14, 15, 16, 17). Extracts were made with 2-ml portions of 10% acid. Particular care was taken to establish a fixed routine for removing the ganglion. The rostral end of the ganglion was freed first and the last step was to cut the preganglionic trunk, so that in stimulation experiments the ganglion remained active until the last possible moment. The time from the beginning of the dissection until the immersion of the ganglion in the ice-cold extractive was regularly 45 ± 15 seconds. Each ganglion was then finely minced without delay and the suspension was kept at 0° C for 90 minutes. At the end of this time, the fluid was transferred with the aid of 1 ml of saline to a 15-ml centrifuge tube (filtration was unnecessary) and extracted 5 times with 10-ml portions of water-saturated ether. The remaining ether was removed by aeration and the supernatant was used directly for the assay. Control experiments showed that added ACh was quantitatively recovered and that the extracts contained no material other than ACh that affected the assay preparation.

Estimation of Plasma Choline

Free choline in cat plasma was extracted essentially by Bligh's (18) method and acetylated with acetyl chloride as described by Emmelin and MacIntosh

(11). Added choline was found to be quantitatively converted to ACh. Results were expressed in terms of choline chloride. HC-3 when present did not interfere with the estimation of choline.

Assay of ACh

Ganglion extracts, samples of venous effluent, and acetylated plasma extracts were assayed against freshly prepared solutions of ACh chloride (Hoffmann-LaRoche) by the cat's blood pressure method as described by MacIntosh and Perry (16). Each sample was injected at two or more dose levels bracketed by ACh standards. The results of duplicate assays generally agreed to within 10%. Standard solutions of ACh for the assay of perfusates were always made up in the perfusion fluid itself, and the volume injected was always about the same for standard and unknown. These precautions were particularly important in the case of plasma samples, since plasma tended to depress the response to simultaneously injected ACh. When the assay preparation deteriorated, as it often did after prolonged testing, it could usually be resuscitated by maintaining a constant infusion of adrenaline hydrochloride (2–3 $\mu\text{g/kg}$ minute) in isotonic glucose. All values for ACh are given in terms of the chloride.

Time Course of ACh Release

ACh set free at the nerve endings of a perfused ganglion reaches the collecting vessel after a short delay, representing the time required for it to diffuse into the vessels and be carried to the venous cannula. This lag tends to obscure somewhat the time course of ACh release when the efflux rate is changing rapidly. Thus when successive 1-minute samples of effluent were collected at the beginning of a stimulation period, the ACh output usually showed a peak in the second minute and then declined (cf. Perry (5)). Control experiments with brief periods of stimulation indicated that with our perfusion routine the mean lag between release and collection was about $\frac{1}{2}$ minute. In plotting the data for ACh output during prolonged stimulation, therefore, it was assumed that the mean output rate during each period of collection after the first minute indicated the rate at which ACh was being released at a time $\frac{1}{2}$ -minute before the mid-point of the collection period: thus the measured output rate during the second minute was plotted as the instantaneous output rate at the end of 1 minute. Since ACh output changed most rapidly at the outset of stimulation, it was found convenient to collect the earlier samples over shorter periods; the routine adopted in experiments where stimulation lasted an hour was to collect 1-minute samples during the first 5 minutes, then one 5-minute sample, two 10-minute samples, and two 15-minute samples. When the ACh output during a brief period of stimulation was to be determined, the effluent was collected for the duration of stimulation and for an additional 2 minutes to ensure that practically all the released ACh had been washed out.

Synthesis of ACh

The amount of ACh synthesized by a perfused ganglion must be equal to the

total quantity of ACh it discharges into the perfusion fluid corrected for the change in its extractable ACh, on condition that no ACh has been lost by leakage of perfusion fluid or by hydrolysis. We have satisfied ourselves that under the conditions of our experiments no correction need be made for loss by leakage. Emmelin and MacIntosh (11) have shown, further, that in experiments of this sort the enzymatic hydrolysis of released ACh must be small. A little non-enzymatic hydrolysis, however, may occur through the action of H ions when ganglia are being extracted with trichloroacetic acid at 0° or through the action of OH ions when bicarbonate-containing perfusates are stored at 0° before being assayed. The errors arising from either source could hardly be more than a few per cent and would affect the calculated synthesis rate in opposite directions: they have therefore been disregarded. Further evidence that the estimates of ACh content and output are reliable is supplied by the satisfactory agreement between output and loss of ganglionic ACh in experiments in which synthesis was prevented. We have therefore calculated the ACh synthesized by a perfused ganglion by adding its residual ACh content to the total output of ACh (as determined by assay of the successive samples of effluent) and subtracting the ACh content of the unperfused control ganglion.

Cholinesterase Inactivation In Vivo

Some experiments were undertaken with the purpose of measuring changes in the ACh content of unstimulated ganglia whose cholinesterase was inactivated as completely as possible for periods of up to 1-2 hours while the natural blood supply was maintained. More satisfactory results were obtained with TEPP than with eserine. Each animal was placed on artificial respiration and given atropine sulphate (1 mg/kg) and gallamine triethiodide (5 mg/kg); the control ganglion was then removed, and the preganglionic trunk supplying the test ganglion was cut or kept in contact with procaine hydrochloride (1%). Treatment with TEPP by vein was then started; the priming dose was 2 mg/kg and a further 1 mg/kg was given every 5 minutes. ACh appeared in the blood of animals so treated and rose within 30 minutes to 10^{-8} g/ml or higher; most of this presumably came from the alimentary canal, since in eviscerated animals similarly treated the blood ACh level stayed below 10^{-9} g/ml. In either case, although blood flow through the ganglion of such an animal was only 0.04-0.10 ml/minute, its rate of ACh release during preganglionic stimulation was about the same, in $\mu\text{g}/\text{minute}$, as for ganglia perfused with eserinated plasma. Transmission through a ganglion so treated and repetitively stimulated failed within a minute, no doubt because of the very high concentration of free ACh ($>10^{-7}$ g/ml) which must have been present in its extracellular fluid. These results were considered to justify the assumption that almost complete inactivation of ganglionic cholinesterase had been achieved by treatment with TEPP.

Results

1. ACh METABOLISM OF RESTING GANGLIA

(A) ACh Content of Resting Ganglia

The ganglia of different cats vary rather widely in ACh content. In a series of 50 resting ganglia we found a mean of 266 μg with a standard deviation of 41 μg . Left and right ganglia from the same cat, however, agree within a few per cent (15). A resting unperfused ganglion may therefore be taken without serious error to contain the same amount of ACh as its fellow did before being perfused.

On this basis resting ganglia perfused for 1 to 2 hours with *eserine-free Locke* or plasma were found to show no important change in their ACh content. This also happened in one experiment in which the hemicholinium base HC-3 ($2 \times 10^{-8} M$) was present in the perfusion fluid. It is possible that in some of these cases a change did occur but was disregarded as falling within experimental error: if so it could hardly have exceeded 10%.

Perfusion of a ganglion with *eserinized* ($8 \times 10^{-6} M$) *Locke*, however, regularly increased the yield of extractable ACh by about 25% (Table I). The effect appeared to be independent of the duration of the perfusion over the range

TABLE I
Effect of perfusion on ACh content of unstimulated ganglion

Perfused with:	ACh as % of ACh in control ganglion		
	Perfused for:		
	5 min	60 min	120 min
Locke	—	100	—
Locke + HC-3	—	100	—
Plasma	—	96	—
Locke + eserine	123 \pm 8 (6)	—	134, 123
Locke + HC-3 + eserine	125 \pm 8 (4)	—	—
Plasma + eserine	129 \pm 13 (3)	193 \pm 11 (3)	202
Locke + eserine at 0° C	*102 \pm 5 (4)	104	—
Plasma + eserine, followed by 15 minutes with plasma alone	—	114, 102	—

NOTE: () Number of observations.

*Perfusion for 5 minutes with eserine-free Locke followed by 15 minutes with eserine-Locke.

5 minutes to 2 hours, and was also seen when HC-3 was present. Brief (5 minutes) perfusion with *eserinized* plasma had a similar effect. This effect of *eserine* could be explained in either of two ways. First, the ACh content of the *uneserinized* control ganglion would be underestimated if the extraction procedure allowed some destruction of ACh by cholinesterase before that enzyme could be inactivated by the extracting fluid. Alternatively, the ACh content of the perfused and *eserinized* ganglion would increase if ACh were formed in it during the first few minutes of perfusion and were preserved from enzymatic

destruction. (The absence of a further increase during continued perfusion of eserinated Locke could then be ascribed either to the exhaustion of some material necessary for the rapid synthesis of ACh, or to saturation of the sites in which the additional ACh was held.) The first alternative seems unlikely because enzymatic destruction of ACh at 0° is a slow process, and because extraction of brain with trichloroacetic acid even at room temperature has been found to yield about the same amount of ACh as extraction at a temperature below 0° (17). Furthermore we have found that if a ganglion is perfused for 15 minutes with ice-cold Locke containing eserine, it yields no more ACh on extraction than its unperfused and uneserinated control (Table I). We conclude therefore that when a ganglion is eserinated at body temperature its extractable ACh is rapidly increased through synthesis by about 25%, and that the ACh extractable by our method from a control ganglion gives a valid measure of normal ACh level at rest.

When *eserinated plasma* was used for perfusion, the ACh content was again increased through synthesis, and during the first 5 minutes to about the same degree as when eserinated Locke was used (Table I). Continued perfusion with eserinated plasma led, however, to a further rise in ACh content: in three experiments the increment in an hour was $93 \pm 11\%$ (mean \pm standard deviation). A similar increase of bound ACh, $93 \pm 20\%$ in an hour, was observed in four ganglia with intact blood supply when TEPP was given repeatedly by vein in large doses (see Methods). The time course of the ACh accumulation is shown in Fig. 1, in which the upper curve is derived from the data of both plasma-perfusion and intact-circulation experiments. In both cases the ACh content, after the rapid initial ascent, rose linearly for an hour and then became steady. This secondary rise of ACh content was not seen in the ganglia of animals treated with both TEPP and HC-3. In this case (as with eserine-Locke perfusion) only the small initial rise appeared, as the lower curve of Fig. 1 shows. It will be shown later that both HC-3 and perfusion with Locke interfere with the ganglion's ability to maintain a high rate of ACh synthesis. In animals treated with HC-3 alone, there was a moderate fall in ACh which became significant ($28 \pm 12\%$) in three experiments lasting 2 hours.

The extra ACh, which accumulates in a ganglion whose cholinesterase has been inactivated, will be referred to as "surplus ACh", and its significance will be discussed later. We may note here, however, that it cannot be free in the ganglion's extracellular spaces, for in that case it would certainly diffuse into the eserinated plasma perfusing the ganglion in amounts that could easily be assayed; and moreover its local concentration would exceed 10^{-5} g/ml, more than enough to produce a complete depolarization block: but there is no block in ganglia so treated, nor do they leak ACh except in minute amounts. A further significant fact is the following. When, after an hour of perfusion with eserine-containing plasma, the perfusion is continued for 15 minutes with eserine-free plasma, most of the surplus ACh is found to have disappeared (Table I). Surplus ACh thus accumulates in some intracellular compartment

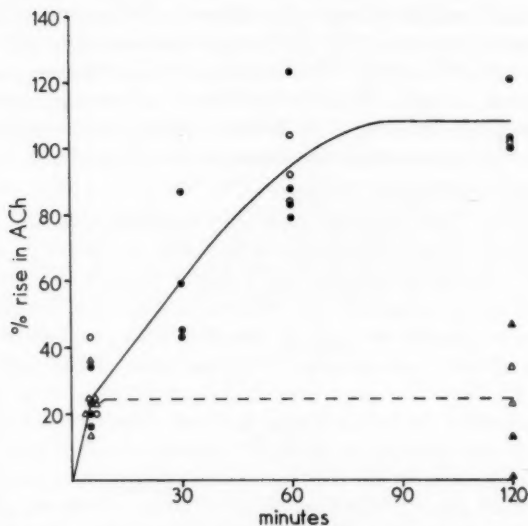


FIG. 1. ACh accumulation in resting ganglia after inactivation of cholinesterase. ●, TEPP, natural circulation; ○, eserine, plasma perfusion; △, TEPP + HC-3, natural circulation; ▲, eserine, Locke perfusion.

where it would have been exposed to enzymatic destruction if the cholinesterase there had not been inactivated. It must be separated in some way from the resting ganglion's normal ACh stock, which is not exposed to the action of the enzyme. The formation of surplus ACh must take place in the region of the nerve endings, rather than in the intraganglionic parts of the cholinergic axons, for the ACh content of the undissected preganglionic trunk was found to increase much less ($22 \pm 10\%$ in nine experiments) than the ACh content of the ganglia, in the experiments in which the whole animal was kept for $\frac{1}{2}$ to 2 hours under the influence of large doses of TEPP.

(B) ACh Release by Resting Ganglia

Earlier workers (10, 19, 20) have stressed their finding that the venous fluid leaving an eserinizated perfused ganglion normally contains very little ACh, and we can confirm this. The rate at which a ganglion perfused with eserinizated plasma, for example, discharges ACh at rest is very small compared with the rate at which it can accumulate surplus ACh, or can discharge ACh when its preganglionic fibers are excited. There is, however, a continuous discharge of ACh from the eserinizated resting ganglion. It amounts to about 0.15–0.5 $\mu\text{g}/\text{minute}$, and so can only be detected when the pooled effluent from several minutes' perfusion is injected into a sensitive assay cat. This minute trickle of ACh is observed also in Locke-perfused ganglia, and continues with little change during at least 2 hours of perfusion. It may well represent a spon-

taneous "quantal" release of the transmitter, analogous to that occurring at the neuromuscular junction (21), but we have not tried to prove that it originates from the axonal endings. The rapidly waning discharge of ACh, occasionally observed at the outset of perfusion in previous studies (1), and attributed (19) to the leaching of ACh from severed nerve trunks, was seldom, if ever, seen in our experiments on plasma-perfused ganglia.

(C) ACh Turnover in Resting Ganglia

There is thus, in a ganglion perfused with eseriniz plasma, a considerable metabolism of ACh, even when no nerve impulses are arriving. A little ACh is being released into the perfusate, and a larger amount (in the first hour of perfusion) is being synthesized and retained within the ganglion as surplus ACh. It seems reasonable to suppose that this ACh metabolism is associated with the preganglionic endings, which contain most of the ganglion's choline acetylase (15, 22, 23). The amount of ACh formed per minute (except for the first few minutes when the rate is faster) is of the order of 1 to 2% of the resting content, and of this perhaps a tenth is released. Such a rate of release, if maintained, would require the resting nerve endings to replace their bound ACh every 8 to 16 hours. It will be shown that a stimulated ganglion under optimal conditions may turn over its bound ACh in about 10 minutes. (These statements refer to ganglia that are perfused with plasma, or retain their natural blood supply. Resting ganglia perfused with Locke form less surplus ACh, but they appear to release ACh at about the same very slow rate.)

In these experiments, synthesis and release of ACh by resting nerve endings were studied in ganglia whose cholinesterase had been inactivated. It seems likely that the same processes occur at similar rates in ganglia not so treated, but cannot then be detected by direct assay because the newly formed or released ester is immediately hydrolyzed.

2. ACh METABOLISM OF ACTIVE GANGLIA

(A) ACh Release by Active Perfused Ganglia

In a series of 20 experiments, four perfusion fluids were compared for their ability to support the ACh metabolism of ganglia subjected to prolonged stimulation. These fluids were: Locke's solution of the usual formula, containing glucose, bicarbonate as the only buffer, and eserine sulphate (8×10^{-6} M); the same solution with the addition of HC-3 (2×10^{-5} M); heparinized cat plasma containing eserine sulphate (3×10^{-5} M); and similar plasma containing HC-3 (2×10^{-5}) as well as eserine. Each fluid had been equilibrated with O_2 , according to the practice of earlier workers, and its pH was therefore about 8.5, or that of an aqueous bicarbonate solution; errors due to enzymatic hydrolysis of ACh in the collected effluent were minimized by chilling the samples and assaying them within 1 to 2 hours. The perfusion fluids were assigned to the experiments on the basis of a randomized block design, with five experiments for each fluid; the procedures for perfusion, stimulation, and assay were kept constant throughout the series.

In each experiment a control sample of effluent, which never contained more than the trace of ACh that corresponds to the resting output, was collected for a 5-minute period before the start of stimulation. The preganglionic trunk was then excited maximally at 20/second for 60 minutes, during which time the whole of the effluent was collected in successive samples and assayed. At the end of stimulation the perfused ganglion was quickly removed and minced in the chilled extracting medium; lastly the opposite ganglion was also removed and similarly extracted. From the assay values, ACh output rates and ganglion contents were calculated, as described under Methods.

The results within each group of five experiments were in good agreement. The time course of ACh output is shown in Fig. 2. The rate of output was

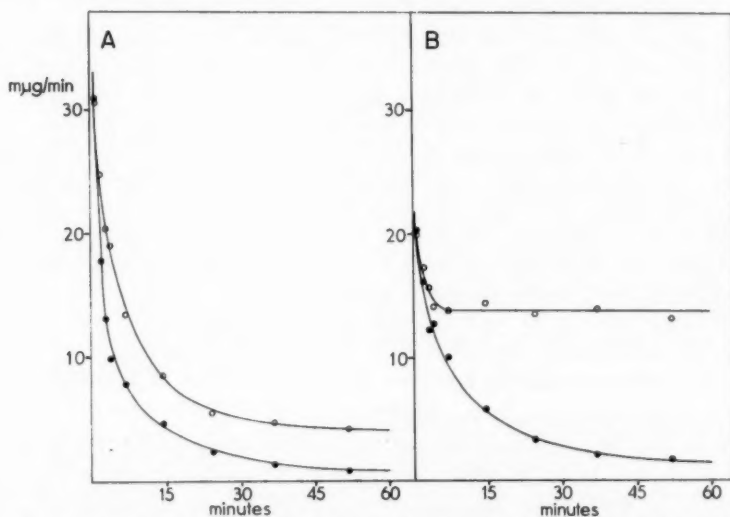


FIG. 2. ACh output of ganglia perfused (A) with oxygenated Locke (eserine $6 \times 10^{-6} M$), (B) with oxygenated plasma (eserine $3 \times 10^{-5} M$). Preganglionic stimulation at 20/second throughout. In each case upper curve is mean of five experiments without HC-3; lower curve is mean of five experiments with HC-3 ($2 \times 10^{-5} M$).

nearly always highest in the sample collected during the 2nd minute of stimulation, and lowest in the sample collected during the last 15 minutes of stimulation. Mean values for these two samples and for the total output, with the corresponding standard deviations, are given in Table II.

(a) *Ganglia Perfused with Locke* (Table II, A)

The five experiments of this group gave results very much like those obtained by previous workers (1, 4, 5). The ACh output rose steeply to a peak (usually for the second 1-minute sample) of 31 ± 2 myug/minute and then fell off gradually towards a much lower level, almost, but not quite, reached during the last 15-minute sample, for which the rate was 4.3 ± 0.7 myug/minute. When the

data were plotted against time (Fig. 2), allowance was made for the time taken for released ACh to reach the collecting vessel, as described under Methods. The above figures for ACh output agree very well with those in a series of experiments by Perry (5), who found 34 ± 4 and 4.0 ± 1.2 $\mu\text{g}/\text{minute}$ for the peak and final output rates. Perry stimulated at 10/second as against 20/second in our tests; but under these conditions of perfusion, as he was able to show, the minute output is nearly independent of the frequency of stimulation, at least over the range 5–100/second.

The total amount of ACh released by these five ganglia during an hour's stimulation averaged 464 μg . This was about double the average initial content, as determined by extraction of the control ganglia.

(b) *Ganglia Perfused with Locke Containing HC-3* (Table II, B)

The ganglia perfused with this fluid discharged ACh, when stimulated, at the same initial rate as the ganglia perfused with Locke. They showed, however, even less ability to maintain a high rate of ACh output (Fig. 2A). The minute output fell off more steeply than in the Locke experiments to a final mean value of 0.9 $\mu\text{g}/\text{minute}$, significantly lower ($P < 0.01$) than the figure for the unpoisoned ganglia, and indeed not much above the threshold for assay. The ability of ganglia poisoned with HC-3 to transmit impulses runs parallel to their ability to release ACh. At the outset of stimulation impulses are transmitted normally; after prolonged stimulation there is little or no transmission, even though eserine is present.

The total quantity of ACh released by these ganglia averaged 246 μg , which was significantly less ($P < 0.01$) than in the experiments with Locke alone, and was only slightly less than the initial store of ACh in the ganglia.

(c) *Ganglia Perfused with Plasma* (Table II, F)

The ganglia of this group did not behave like those perfused with Locke. Their peak rate of ACh output averaged 20 $\mu\text{g}/\text{minute}$, which was significantly ($P < 0.01$) lower than that of the Locke-perfused ganglia (Fig. 2B). The output, however, was better maintained than in the Locke experiments. It fell off by only about one-third, and this fall was complete within the first 5 minutes, after which the rate remained practically steady. The output for the final sample, 13 $\mu\text{g}/\text{minute}$, was significantly higher ($P < 0.01$) than in the Locke experiments.

The total output of ACh from these ganglia was 852 μg , significantly ($P < 0.01$) greater than in either of the preceding series, and more than 3 times the initial ACh content of the ganglia.

Emmelin and MacIntosh (11) compared the ACh output rates of Locke- and plasma-perfused ganglia during 3-minute periods of stimulation and found that they were about the same. In fact, they reported somewhat higher figures for their Locke than for their plasma experiments, and though they did not regard the difference as significant, their results are clearly in satisfactory agreement with our own.

(d) *Ganglia Perfused with Plasma Containing HC-3* (Table II, G)

The five ganglia perfused with this fluid released ACh at the same initial rate as those in the preceding series (Fig. 2B). Thereafter they behaved like the ganglia perfused with Locke containing HC-3. Their ACh output fell off steeply and continued to fall: the mean rate for the final sample, 1.8 m μ g/minute, though significantly ($P < 0.05$) above the mean for the experiments with Locke containing HC-3, was far below the mean for the experiments with Locke alone.

The total output of ACh from these ganglia, 285 m μ g, was a little higher than their mean initial content, but not significantly greater than the total output from ganglia perfused with Locke containing HC-3.

Thus, with prolonged stimulation, plasma-perfused ganglia release at the outset less ACh than Locke-perfused ganglia; but this is true for only a few minutes: thereafter their ACh output stabilizes at a much higher level, unless HC-3 is present. The effects of HC-3 are the same with either fluid: the initial rate of ACh output is not altered; but the output, instead of declining only to a characteristic steady level, falls almost to zero. It is clear that these findings can be most simply explained by supposing (a) that ACh is more readily released, from the preformed stock at the axon endings, when Locke rather than plasma is used for perfusion; but that (b) in the presence of plasma this stock (and therefore the ACh output rate) is better maintained during stimulation, because ACh can be synthesized faster; while (c) HC-3 prevents ACh synthesis, so that both stock and output fall to low levels if stimulation is prolonged. The correctness of these suppositions is proved by the observations described in the next section.

(B) *ACh Turnover in Active Perfused Ganglia*

Each of the 20 ganglia whose ACh output was followed during an hour's stimulation was excised at the end of that time, and its ACh content was compared with that of its unperfused control. The difference between these two values, combined with the output of ACh into the perfusate, gave the amount of ACh synthesized by the perfused ganglion. The data are summarized in Table II.

(a) *Ganglia Perfused with Locke*

The five ganglia perfused with Locke (Table II, A) were found to have lost about half ($53 \pm 11\%$) of their preformed ACh as a result of being stimulated for an hour. In view of the waning ACh output in these experiments the loss would not have been surprising, were it not that previous workers failed to obtain it. Brown and Feldberg (1), who did four experiments of this sort, found no significant change in ACh content: their stimulated ganglia appeared sometimes to lose, and sometimes to gain, ACh. Kahlson and MacIntosh (4) in three tests did find a loss but it was only 24%. Our lack of agreement with the earlier workers on this point is the more puzzling, since our figures for ACh output are so similar to theirs. It is true that they stimulated at rather lower

TABLE II
ACh turnover in perfused ganglia
(mean \pm S.D.)

Group	Perfusion fluid	No. of expt.	Gas	Eserine ($M \times 10^{-6}$)	ACh output			ACh content			ACh synthesis	
					1-2 min (mg/min)	45-60 min (mg/min)	Total (mg)	Initial (mg)	mg	% of initial	Total (mg)	Rate (mg/min)
A	Locke	5	O ₂	8	30.6 \pm 2.4	4.3 \pm 0.7	464 \pm 61	241 \pm 26	111 \pm 22	47 \pm 11	334 \pm 67	5.6 \pm 0.9
B	Locke + HC-3 (2×10^{-3} M)	5	O ₂	8	31.0 \pm 4.6	0.9 \pm 0.4	246 \pm 47	250 \pm 85	48 \pm 14	19 \pm 3	44 \pm 72	0.7 \pm 1.2
C	Locke	3	O ₂ + CO ₂	8	30.7* \pm 7.0	7.2 \pm 1.0	610 \pm 57	310 \pm 22	170 \pm 46	55 \pm 13	470 \pm 80	7.8 \pm 1.3
D	Locke + choline (3.5×10^{-3} M)	3	O ₂ + CO ₂	8	26.3* \pm 5.5	11.8 \pm 0.7	733 \pm 89	238 \pm 35	493 \pm 97	207 \pm 35	988 \pm 131	16.5 \pm 2.1
E	Locke	5	O ₂	40	31.8 \pm 3.7	2.5 \pm 0.8	367 \pm 67	321 \pm 89	166 \pm 81	51 \pm 15	212 \pm 68	3.5 \pm 1.1
F	Plasma	5	O ₂	40	20.0 \pm 4.5	13.2 \pm 3.5	852 \pm 114	265 \pm 48	593 \pm 101	224 \pm 43	1180 \pm 141	19.7 \pm 2.4
G	Plasma + HC-3 (2×10^{-3} M)	5	O ₂	40	20.4 \pm 5.7	1.8 \pm 0.5	285 \pm 103	237 \pm 56	61 \pm 20	26 \pm 7	109 \pm 98	1.8 \pm 1.6
H	Plasma	5	O ₂ + CO ₂	40	31.0 \pm 6.6	27.8 \pm 7.7	1591 \pm 438	267 \pm 51	397 \pm 105	151 \pm 35	1721 \pm 344	28.7 \pm 6.8

*In experiments of groups C and D, peak outputs sometimes occurred in 0- to 1-minute sample.

frequency than we did, 10–17/second as against 20/second, but in view of Perry's results (5) it seems quite unlikely that this difference can account for the discrepancy. It is easier to suppose that in the present experiments we were more successful in continuing to stimulate the ganglion effectively right up to the moment of its excision and immersion in extracting fluid: for there is evidence (4, 24) that the ACh stores of nervous tissue when depleted by activity can be made good by a brief period of rest. Whether or not this is the explanation of our failure to confirm the older results, we feel sure that the present observations cannot be seriously in error. We have repeated our experiments, using a higher frequency of stimulation, or a different anticholinesterase drug, or one of several modifications of the Locke's solution formula, and have always obtained the same result.

We can, however, confirm the finding of Brown and Feldberg (1) that the Locke-perfused ganglion synthesizes ACh during preganglionic stimulation. The total amount formed when the stimulation lasted an hour exceeded the initial ACh content of the ganglion; it was less than the total amount discharged into the perfusate, since some of this total was accounted for by expenditure of the ganglion's ACh stores. Figure 3 presents the balance sheet in diagrammatic form, with the results for Locke perfusion on the left. If it is assumed that synthesis proceeded at a constant rate throughout the period of stimulation, one would have expected the calculated rate of synthesis to be about equal to the rate of ACh discharge observed at the end of the period, at a time when synthesis and release have come nearly into balance. This expectation was only approximately realized: the mean rate of synthesis as derived from the data for ACh output and change in ACh content was 5.6 ± 0.9 $\mu\text{g}/\text{minute}$, significantly higher ($P < 0.05$) than the mean output rate for the last 15-minute sample (4.3 ± 0.7 $\mu\text{g}/\text{minute}$), which was itself no doubt a little higher than the final asymptotic rate of release. Several possible explanations may be suggested for this small but real discrepancy. In the first place, it may be noted that each ganglion at the beginning of stimulation must have contained some 55 μg of ACh that was not present at the beginning of perfusion: this was the surplus ACh formed during the 15 minutes in which the ganglion was perfused with eserinizied fluid but not stimulated. The amount of surplus ACh present at the end of the experiment is not known: it might have been higher, as a result of continued formation, or lower, as a result of dissipation. If the same amount were present at the end of stimulation as at the beginning the calculated rate of ACh synthesis would have been 4.7 $\mu\text{g}/\text{minute}$, not much above the observed final rate of ACh release. Alternatively, there may have been some falling-off in the rate of ACh synthesis during the hour of stimulating, for instance because of a diminishing supply of free choline. Or a little ACh may have been formed during the unavoidable delay between the excision of the ganglion and its fixation in the cold extracting fluid. In any case it seems reasonable to suppose, on the basis of the data presented above, that the final steady rate of ACh release, in a Locke-perfused ganglion subjected to prolonged

stimulation, is a measure of the rate at which the ganglion is synthesizing ACh.

Perry (5) found that a Locke-perfused ganglion, which had been stimulated for a long time in the absence of eserine, could on being eserinizied release as much ACh as if it had not been active. On the basis of this and other evidence he concluded that although the nerve endings cannot recapture the ACh they have released, they can take up the choline derived from its hydrolysis and use it to manufacture new ACh. If this can happen one would expect that when two ganglia, one eserinizied and one not, were perfused with Locke and stimulated for the same time, the eserinizied ganglion would show a greater loss of bound ACh. Experiments to test this idea did not give a conclusive answer. Three ganglia perfused with eserine-free Locke and stimulated for an hour lost $38 \pm 15\%$ of their ACh. The loss was significant ($P < 0.05$), but not significantly smaller ($P > 0.1$) than the $53 \pm 11\%$ lost by the five ganglia stimulated during perfusion with eserine-Locke. Further testing might have shown the difference to be a genuine one. It is also possible that some of the ACh remaining in the eserinizied ganglion at the end of stimulation was surplus ACh formed earlier in the experiment: the presence of surplus ACh would tend to obscure any greater depletion of the regular stock of ACh that might have occurred under the influence of eserine. It seems clear, in any case, that even in the absence of eserine a Locke-perfused ganglion cannot synthesize ACh as fast as it can release it, and consequently suffers some diminution of its transmitter stocks. It will be shown later that this deficiency of ACh synthesis, characteristic of the Locke-perfused ganglion, can be corrected by adding choline to the perfusion fluid.

(b) Ganglia Perfused with Locke Containing HC-3

The five ganglia perfused with this fluid lost on the average 81% of their ACh during an hour's stimulation (Table II, B). This was a significantly greater loss ($P < 0.01$) than that found in the ganglia perfused with Locke without the drug. It was accounted for, within the limits of experimental error, by the ACh that appeared in the perfusate (Fig. 3): the calculated mean rate of synthesis, 0.7 ± 1.2 m μ g/minute, though about equal to the mean final rate of ACh discharge, was not significantly different from zero. Thus the inability of these ganglia to replace their lost ACh was reflected both by the greater depletion of their ACh stores as compared with Locke-perfused ganglia, and also by the almost complete cessation of ACh discharge by the end of the experiment.

Since stimulation of a ganglion perfused with eserinizied Locke, especially in the presence of HC-3, can lead to so great a loss of its ACh, it seems clear that the extractable ACh of the ganglion, or at any rate the greater part of it, must represent the depots from which ACh is set free when nerve impulses reach the synapses. It is unnecessary to consider, as Brown and Feldberg (1) felt obliged to do, the possibility that each impulse brings about the synthesis of the ACh it releases. But although most of the ganglion's preformed ACh

can be set free by sufficiently prolonged excitation, it must be pointed out that we have never been able in this way to deplete a ganglion completely of its ACh. The residual content, after an hour's stimulation in the presence of HC-3 had reduced the ACh output to a very low level, was 48 ± 14 μg , or about 19% of the original amount. Still longer stimulation might have reduced it a little further, say to 40 μg , but hardly to below that figure.

It might have been thought that this residual ACh represented some of the surplus ACh which must have been formed in these experiments during the 15 minutes of perfusion with eserinizd fluid that preceded the hour of stimulation. On the basis of our results with unstimulated ganglia (Table I) there should have been some 55 μg of ACh in this fraction at the end of perfusion, more than enough to account for the 48 μg actually found in the ganglia. We have, however, found in parallel experiments, in which ganglia were stimulated for an hour while being perfused with Locke containing HC-3 but no eserine, that the residual ACh was not significantly less than when eserine was present: the final contents in two trials were 36 and 45 μg . It must therefore be concluded that surplus ACh disappears, even in the presence of eserine, where ACh synthesis is defective. The ACh left in a ganglion, during prolonged stimulation under such conditions, must constitute a different fraction of the ganglion's extractable ACh. Since the ACh in this fraction is not releasable by stimulation, we shall designate it 'stationary ACh', in contrast to the 'depot ACh' which accounts for most of the ganglion's resting content and represents the stock available for synaptic transmission.

The ACh of the preganglionic trunk behaves like stationary ACh. Its concentration (average 35 $\mu\text{g/g}$ wet weight) is not appreciably altered by prolonged stimulation of the trunk in the whole animal, even when HC-3 has been given in massive dosage. The intraganglionic portions of the preganglionic fibers must compose at least 4% of the bulk of the ganglion, and more than that if the total cross section of these fibers increases with their branching. If the axonal ACh concentration were the same inside as outside the ganglion, the intraganglionic portions would account for at least 20 μg of the 40 μg in the stationary fraction. A little ACh is doubtless present also in cholinergic axons originating within the ganglion. It seems reasonable to conclude that nearly all the stationary ACh is extrasynaptic.

(c) Ganglia Perfused with Plasma

We have shown that ganglia perfused with eserinizd plasma, and not poisoned by HC-3, can maintain a relatively high rate of ACh output however long they are stimulated. It would therefore be expected that their ACh stores would remain at a correspondingly high level. This has been confirmed. In fact, as Table II, F, shows, such ganglia actually accumulate ACh during prolonged stimulation: the mean percentage increase of ACh content in the five experiments amounted to 124 ± 43 . This increase was not significantly different from the $93 \pm 11\%$ found for resting ganglia perfused for the same time

with eserinizied plasma, and, like it, must be thought to represent 'surplus' ACh, held in some location other than the depots in which releasable ACh is normally stored. Because in plasma-perfused ganglia the ACh output remains steady while the ganglion is nearly doubling its ACh content, we must conclude that surplus ACh does not contribute substantially to the ACh output during synaptic activity.

ACh was synthesized in these experiments (Fig. 3) at an average rate of about 20 $\mu\text{g}/\text{minute}$, 3 or 4 times as fast as in parallel trials with Locke perfusion. Since, however, the plasma-perfused ganglia had a significantly lower initial rate of ACh output, they must have been less efficient in releasing their stored ACh. It will be shown later that this is an abnormality that can be corrected by raising the CO_2 tension of the perfused plasma.

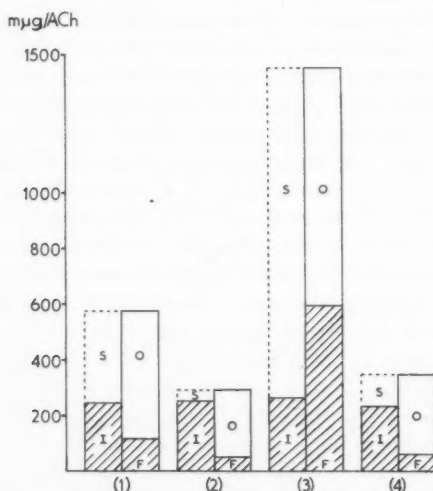


FIG. 3. ACh turnover in perfused ganglia: same experiments as in Fig. 2. I, initial ACh content; F, final ACh content; O, total output of ACh during stimulation for 1 hour; S ($= F + O - I$), ACh synthesized during experiment.

(1) Ganglia perfused with Locke. (2) Ganglia perfused with Locke + HC-3. (3) Ganglia perfused with plasma. (4) Ganglia perfused with plasma + HC-3.

(d) Ganglia Perfused with Plasma Containing HC-3

These ganglia, like the Locke-perfused ganglia exposed to HC-3 in the same concentration, were found to have lost most of their ACh during an hour's stimulation (Table II, G). The mean loss, $74 \pm 7\%$, was somewhat, but not significantly, less than in the experiments with Locke and HC-3, and the calculated rate of synthesis, $1.8 \pm 1.6 \mu\text{g}/\text{minute}$, was not significantly greater than zero ($P > 0.05$, < 0.1). Thus even in ganglia perfused with plasma, which in the absence of HC-3 supports ACh synthesis so much better than Locke does, the effect of HC-3 was to inhibit ACh synthesis almost completely.

There is a suggestion, however, that a little more ACh was manufactured than in the experiments with Locke and HC-3.

(C) ACh Turnover in Active Ganglia with Intact Blood Supply

(a) Effect of Prolonged Stimulation on ACh Content

The high steady rate of ACh discharge in plasma-perfused ganglia subjected to prolonged stimulation suggested that the depot ACh under these conditions also stayed at a high steady level. The correctness of this idea could be tested by measuring the ACh content of ganglia supplied with blood or plasma and stimulated in the absence of any anticholinesterase drug, since any change in the depot ACh should then not be obscured by the accumulation of surplus ACh. Three experiments of this sort have been done. When one of a pair of ganglia, both with their blood supply intact, was stimulated for 1-2 hours at 20/second while the other remained at rest, their ACh content at the end of the period differed by less than 5%. No indication was found of the sequences of changes described by Rosenblueth, Lissák, and Lanari (25) in the ACh content of ganglia subjected to prolonged repetitive excitation; but we stimulated at a lower frequency and did not try to reproduce their experimental conditions exactly. Our own data thus show that ganglia stimulated repetitively at frequencies up to 20/second maintain their depot ACh at close to its resting level for at least an hour or two, provided that they are supplied with the normal blood (or plasma) necessary for efficient ACh synthesis.

(b) Effect of HC-3 on ACh Content and Release

In the experiments with HC-3 that have been described above (*B*, (*b*) and (*d*)), the ganglia could be considered abnormal both because they were perfused and because they were under the influence of eserine. But the effect of HC-3 on ACh synthesis does not depend on the existence of such abnormal conditions, for we have found that ganglia that were neither perfused nor eserinizd behaved similarly, in that they lost their stored ACh when they were stimulated in the presence of HC-3. In five experiments on cats that had been given no drug but chloralose, and were maintained on artificial respiration, the preganglionic trunk of one side was stimulated at 20/second for 20 minutes and the ganglion was immediately removed for extraction. HC-3 (1 mg/kg) was then given by vein and the second ganglion was similarly stimulated. All the unpoisoned control ganglia were found to contain the usual amount of ACh, but the ganglia stimulated after injection of HC-3 contained only $29 \pm 9\%$ as much as their fellows. In these experiments the percentage loss of ACh was a little less than in the earlier experiments in which HC-3 was added to the perfusion fluid, but in the earlier tests the stimulation had lasted 3 times as long.

(c) Delayed Ganglion Block after HC-3

In the experiments just described the ACh output of the-stimulated ganglia could not be measured directly, but indirect evidence was obtained that the output fell off under the influence of HC-3, just as it had done in ganglia that were perfused and eserinizd. At the outset of preganglionic stimulation, the

retraction of the nictitating membrane, when the drug had been given, matched the retraction that had previously been obtained from the opposite membrane. But while the latter had responded maximally right through the 20 minutes of

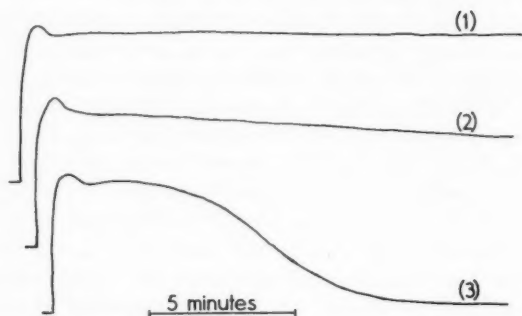


FIG. 4. Responses of cat's nictitating membrane to continued sympathetic stimulation at 20/second: (1) before HC-3, preganglionic stimulation; (2) after HC-3 (1 mg/kg), post-ganglionic stimulation; (3) after HC-3, preganglionic stimulation.

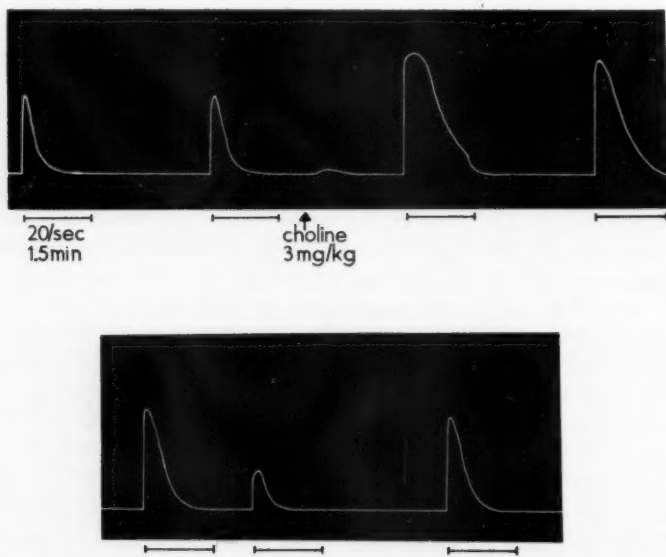


FIG. 5. Partial restoration by rest and by choline of ganglionic transmission in cat poisoned by HC-3. Nictitating membrane: bars indicate maximal preganglionic stimulation for 1.5 minutes at 20/second; except in penultimate case, interval between stimulation periods was 2.5 minutes. Before the record started, the cat had been given HC-3 (3 mg/kg) and placed on artificial respiration, and complete ganglionic block had been produced by stimulating the preganglionic trunk for 20 minutes. At arrow, choline chloride (3 mg/kg) by vein.

stimulation, the response in the poisoned animal always began to fall off within a few minutes and eventually disappeared almost completely. With post-ganglionic stimulation, on the other hand, a nearly maximal retraction of the membrane could be maintained in the poisoned animal for at least an hour (Fig. 4). In further experiments it was shown that the response to preganglionic stimulation, when it had failed, could be partially restored either by rest, or by lowering the frequency of stimulation, or by injecting a small dose of choline chloride (1-4 mg/kg). Such a dose of choline, in the absence of HC-3, produces no more than a small and fleeting effect either on ganglionic transmission or on the nictitating membrane itself (Fig. 5).

The possibility that HC-3 might possess slowly developing ganglion-blocking activity, or might be converted *in vivo* into a ganglion-blocking agent, was eliminated by the observation that even after repeated large doses of the compound had been given the ganglion transmitted impulses normally when it was first subjected to preganglionic stimulation, and failed to transmit only when it was stimulated repetitively for several minutes. HC-3 has been found to possess some activity as a neuromuscular blocking agent (12), but if it has any ganglion-blocking power this must be weak: in one test on a perfused uneserinized ganglion we found that it actually sensitized the ganglion cells to the action of exogenous ACh. So far as our observations go, HC-3 has no important effect on autonomically innervated structures that cannot be explained by its ability to inhibit ACh synthesis, and thus to produce delayed failure of transmission at repetitively activated cholinergic junctions.

3. SUBSTANCES THAT INFLUENCE THE ACh METABOLISM OF PERFUSED GANGLIA

It has been shown above that ganglia perfused with plasma synthesize ACh more efficiently but release it less readily than ganglia perfused with Locke. This difference of behavior could not have been due to differences in O_2 tension, perfusion pressure, or flow rate, since these were similar for the two fluids. It is explained at least in part by the experiments described below, which show that ACh turnover in a perfused ganglion may be affected by altering the concentration in the perfusion medium of CO_2 , choline, and (or) an unidentified factor (or factors) present in plasma. The effects of eserine and of adrenaline have also been examined in this connection.

(A) Carbon Dioxide

The Locke and plasma used in the perfusion experiments so far described had been gassed with O_2 and were unphysiologically alkaline (pH up to 8.5). It was thought that in plasma so alkaline there might be a significant reduction of ionized calcium, which is known to be required for the release of ACh at ganglionic (26, 27) and neuromuscular junctions (cf. 28). Such an effect would be greater with plasma perfusion than with Locke perfusion for two reasons: first, the protein and phosphate (and perhaps the heparin) of plasma would tend to bind calcium ions (29, 30); and secondly, in the presence of plasma the

endogenous CO_2 produced by neuronal activity would be less effective in lowering the pH locally, since plasma is better buffered than Locke. A new series of experiments was therefore carried out, in which the pH of each fluid was lowered to 7.4 ± 0.1 (at 38°C) by equilibrating it with O_2 to which the required concentration of CO_2 had been added.

Plasma so treated was used for perfusion in five experiments (Table II, H). In these the initial rate of ACh release was found to be higher than in the earlier experiments with CO_2 -free plasma, and not significantly different from the initial rate in the experiments with Locke. Some decrease of the output rate occurred as usual during the first 5 minutes, but in this case it amounted to only about 10%; and for the remainder of the experiment the output was nearly constant and higher than in any other series of experiments (Fig. 6). The total amount of ACh discharged was 87% greater, and the mean rate of synthesis was 46% greater, than in the tests with CO_2 -free plasma: the difference in both cases was significant ($P < 0.01$ and $P < 0.05$). The ACh content of each ganglion rose during the experiment, but not so far as in the experiments without CO_2 : this difference was also significant ($P < 0.05$). It follows from these observations that the more rapid output of ACh, when CO_2 is present, does not depend on a corresponding elevation of the depot ACh; the primary effect of CO_2 must rather be to facilitate the release of ACh. The more rapid release of ACh is accompanied by an increase in its rate of synthesis, so that the stock of available transmitter is well maintained. But this is a secondary effect. Some mechanism must exist by which synthesis is kept in step with release, as is illustrated by the fact that in an active ganglion the rate of synthesis is higher than in a resting ganglion. In the present experiments, at least, there is nothing to suggest that CO_2 has any important direct action on ACh synthesis.

Experiments to test the effect of CO_2 on the ACh metabolism of Locke-perfused ganglia led to similar conclusions (Table II, C). In these trials the bicarbonate content of the Locke was raised to $2.5 \times 10^{-2} M$ with a corresponding reduction of chloride; the solution was then better buffered and its CO_2 tension was about the same as that of the CO_2 -treated plasma. The ACh output of the three ganglia perfused with this fluid was initially about the same as that of the ganglia perfused with CO_2 -free Locke; thereafter it was somewhat better maintained and the total output was significantly ($P < 0.01$) larger, though it was still significantly below the output from the plasma-perfused ganglia even when these latter were not supplied with CO_2 (Fig. 7). The reduction in ganglionic ACh was about the same as when CO_2 was omitted from the medium. The effects of CO_2 in these experiments on Locke-perfused ganglia, like the more striking effects seen with plasma perfusion, support the idea that CO_2 exerts its action mainly by promoting ACh release rather than by speeding ACh synthesis. It is plausible to suppose, but remains to be proved, that this action depends mainly on increased ionization of calcium.

An apparently similar effect of CO_2 (or the CO_2 -bicarbonate system) in promoting ACh turnover was observed by Quastel, Tennenbaum, and Wheatley

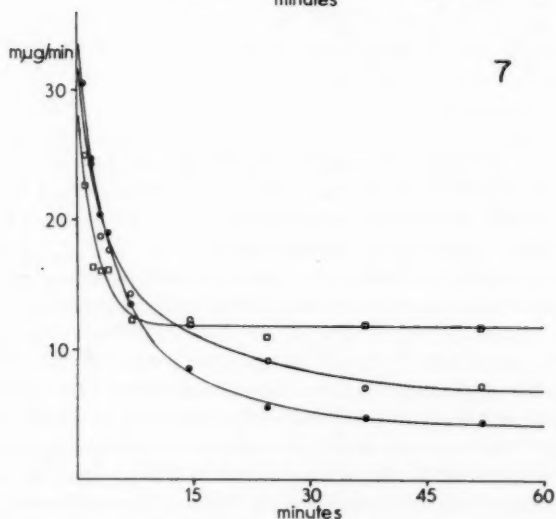
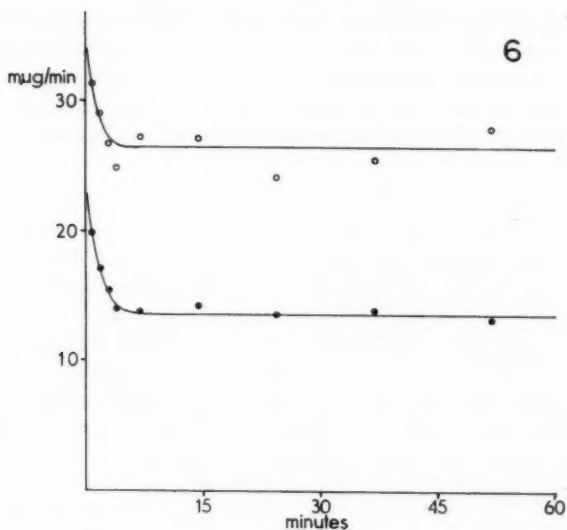


FIG. 6. ACh output of plasma-perfused ganglia. Eserine (3×10^{-6} M); preganglionic stimulation at 20/second throughout. Upper curve, mean of five experiments in which plasma had been gassed with CO_2 - O_2 mixture and was at pH 7.4; lower curve, mean of five experiments in which plasma had been gassed with O_2 only (cf. Fig. 2B).

FIG. 7. ACh output of Locke-perfused ganglia. Eserine (6×10^{-6} M); preganglionic stimulation at 20/second throughout. ●, mean of five experiments with Locke of original formula, gassed with O_2 (cf. Fig. 2A); O, mean of three experiments with high-bicarbonate Locke, gassed with CO_2 - O_2 to pH 7.4; □, mean of three experiments with Locke of latter composition supplemented with choline 3.5×10^{-6} M.

(31) and analyzed by McLennan and Elliott (32), in experiments on brain slices. McLennan and Elliott showed that the effect of CO_2 is a specific one and does not depend merely on lowering the pH of the medium. Emmelin and MacIntosh (11) also concluded from their experiments on Locke-perfused ganglia that the rate of ACh release is little affected by changes in pH over the range 7.4–8.5.

(B) Choline

Brown and Feldberg (1) suspected that choline might be a rate-limiting factor for ACh synthesis in Locke-perfused ganglia. They therefore tested the effect of adding choline to the perfusion fluid at a time when the ACh output had been reduced to a low level by prolonged stimulation. In 5 out of 10 trials they got positive results; and in another experiment, in which choline ($1.4 \times 10^{-5} M$) had been present from the start, the ACh output appeared to fall off more slowly than it usually did. We have done three further experiments of the latter sort. The concentration of choline was $3.5 \times 10^{-5} M$, and the Locke to which it was added contained extra bicarbonate and was equilibrated with $\text{CO}_2\text{--O}_2$ to bring its pH to 7.4, as described in the last section.

The effect of adding choline was unambiguous (Table II, D). The ACh output from the stimulated ganglia began at the usual rate and showed the usual early decline, but became steady within 10 minutes at a level significantly ($P < 0.01$) higher than in the experiments with $\text{CO}_2\text{--Locke}$, though well below the steady level obtained with $\text{CO}_2\text{--plasma}$ (Fig. 7). Even more striking was the effect of choline on the ACh content of the stimulated ganglia. Instead of being reduced by 40–60% as in all the other experiments with Locke perfusion, it rose by 107%, significantly further than in the experiments with $\text{CO}_2\text{--treated plasma}$, and nearly as far as in the experiments with $\text{CO}_2\text{--free plasma}$. These ganglia, therefore, were able to synthesize ACh at a perfectly adequate rate: in this respect they resembled ganglia perfused with plasma. They were, however, unable to release it at the maximal rate: in this respect, and also in their tendency to accumulate a great deal of surplus ACh, they behaved very much like ganglia perfused with $\text{CO}_2\text{--free plasma}$, but unlike ganglia perfused with $\text{CO}_2\text{--treated plasma}$. Since, however, these ganglia were exposed to CO_2 at the same tension as the ganglia perfused with $\text{CO}_2\text{--plasma}$, which were efficient releasers of ACh, we must conclude that plasma contains a factor (or factors) necessary for optimal ACh release, and different from CO_2 . The superiority of $\text{CO}_2\text{--treated plasma}$ over Locke containing choline, as a medium for the support of ACh release, must be due to the presence of this unknown material. But the superiority of plasma over ordinary Locke, as a medium for the support of ACh synthesis, must be due at least in large part to the presence of choline. Bligh (18) found that cat plasma contains about $5 \times 10^{-6} M$ choline, and we have obtained similar values. While this is only one-seventh of the concentration in our choline-enriched Locke, it is apparently enough for optimal ACh synthesis, for we have found that the addition of extra choline to the perfusing

plasma does not appreciably increase the output of ACh during prolonged high-frequency stimulation.

Even in a ganglion perfused with Locke, some free extracellular choline is probably available to the nerve endings for ACh synthesis. Such a ganglion continues to discharge choline at the rate of 10–50 $\mu\text{g}/\text{minute}$, even after long perfusion without stimulation, and whether eserine is present or not (5, 11). This choline may not all come from the region of the synapses, but that some of it does is made likely by an observation of Perry (5). He found that during preganglionic stimulation in the absence of eserine the choline output first rose and then fell: the rise, as he pointed out, might be due to the appearance of hydrolyzed ACh (cf. 1, 11) and the fall, to uptake of extracellular choline for ACh synthesis. Some results of our own also suggest that the active ganglion may draw on a store of endogenous choline. In two experiments we added HC-3 at the usual concentration to the plasma perfusing a ganglion that was being stimulated, and observed, in addition to the expected fall in ACh output, a sharp but temporary increase in the output of choline: the peak rate of discharge, after allowing for the choline originally present in the plasma, was 200–400 $\mu\text{g}/\text{minute}$. HC-3 may perhaps have released this choline through some kind of base-exchange reaction. It seems not unlikely, however, that in the active ganglion choline released from some cellular store is available for the synthesis of ACh, and perhaps also of phospholipid, unless its uptake is prevented by HC-3.

(C) Antagonism of HC-3 by Choline

Schueler (12) showed that choline is an effective antidote to the toxic action of HC-3 in mice. It has since been found that HC-3 does not inhibit ACh synthesis by minced brain if the choline concentration of the medium is raised (6, 33, 34). Choline can also antagonize HC-3 at ganglionic synapses, as we have shown in the experiments already described, in which choline restored transmission through ganglia that had become blocked as a result of stimulation in the presence of HC-3.

Experiments on perfused ganglia gave further evidence that choline acts as an antidote to HC-3 because it supports ACh synthesis, and consequently allows ACh output to be maintained. The perfusion fluid was eserinated Locke or plasma containing HC-3 ($2 \times 10^{-5} M$), and the preganglionic trunk was stimulated at 20/second. When choline was added to the fluid before stimulation began, the ACh output was better maintained than in its absence, and the ganglion lost less of its ACh; when choline was added after the effect of HC-3 was well developed, the ACh output of the stimulated ganglion rose to a higher level and remained there. The degree of restoration of synthesis depended on the choline:HC-3 ratio. When the molar ratio was 5:1 or 50:1 the inhibitory effect of HC-3, though reduced, was still present; when it was 1000:1 the normal rate of synthesis was restored and the ganglia gained ACh while they were being stimulated.

(D) Eserine

Shelley (35) found that eserine in high concentration ($2.7 \times 10^{-3} M$) reduces the rate at which brain slices form ACh. She showed that the effect can be antagonized by choline, and concluded that eserine can compete with choline at the active centers of choline acetylase. Perry (5) also concluded, as we have noted, that eserine inhibits ACh synthesis in perfused ganglia, but he explained its action somewhat differently: he considered that choline, but not ACh, can be taken up by the nerve endings and that eserine affects synthesis by preventing the hydrolysis of released ACh.

We have found that Locke-perfused ganglia synthesize less ACh during stimulation when the concentration of eserine is raised (Table II, A and E). In five experiments eserine was added to the oxygenated Locke at 5 times the usual level: its concentration was then the same ($3 \times 10^{-5} M$) as in the plasma experiments. The initial ACh output matched the output found in the experiments with Locke containing the lower concentration of eserine, but the final output rate and the rate of synthesis were both significantly lower ($P > 0.05$). These results suggest that the inhibitory effect of eserine on ACh turnover was in fact due to an action on ACh synthesis, as Shelley and Perry have proposed, rather than on ACh release. The suggestion would have been verified if the ganglia treated with the higher eserine concentration had lost more of their ACh during stimulation, but in fact the loss was about the same as for ganglia exposed to the lower level of eserine. A less equivocal result might have been found if we had used eserine in 100-fold higher concentration, as Shelley did. In her *in vitro* experiments, as well as in those of Mann, Tennenbaum, and Quastel (36) on the ACh metabolism of brain slices, the distribution of ACh between tissue and medium was not affected by raising the eserine concentration. As Shelley points out, this finding argues against an important inhibitory effect of the drug on ACh release. It seems probable, on the whole, that eserine can interfere with ACh synthesis in two different ways: first, as Perry suggested, merely by preventing the hydrolysis of released ACh to choline; and secondly, by competing with choline in some process necessary for ACh synthesis. One might expect that the first effect would be more prominent at low levels of eserine, and would only be significant in experiments where the perfusion fluid carried no exogenous choline to the ganglion; the second effect would become more important as the concentration of eserine was raised: excess of choline would antagonize either effect. Further work is needed to decide these points. The present experiments at least show that the superiority of plasma over Locke as a medium favoring ACh turnover, in the perfusion experiments described earlier, could not be due to the higher eserine content of the plasma.

(E) Adrenaline

The effect of adrenaline was tested because of the possibility that the plasma used for perfusion contained significant amounts of adrenaline set free by

pressor reflexes in the donor animals. Paton and Thompson (37) have already shown that adrenaline diminishes ACh release in Locke-perfused ganglia. Their finding was confirmed in one experiment, in which the concentration of adrenaline was 5×10^{-8} g/ml and the reduction of output during prolonged stimulation at 20/second was about 50%; no effect was detected when the concentration was 10^{-8} . The opposite result was obtained in some experiments with plasma. After a steady level of ACh release had been established, the addition of adrenaline (10^{-8} g/ml) to the perfusion fluid reversibly increased the output. This effect of adrenaline was not due to vasoconstriction, which was slight or absent: it seemed to be greater when the output rate was low, and was not seen at all in one experiment when the rate was higher than usual. Adrenaline opposes the action of ACh on ganglion cells (37), and most workers have agreed with Marrazzi (38) that it inhibits ganglionic transmission, although it may under some conditions facilitate neuromuscular transmission. A facilitatory action on ganglia may, however, appear under some conditions, especially when the concentration is low (39, 40) and the present observations suggest that this may be due to an increased release of ACh. It is unlikely that the increased release is due to a more rapid synthesis of ACh, for in the plasma-perfused ganglion synthesis is already adequate to maintain the depots fully stocked.

(F) A Plasma Factor that Promotes ACh Release

It has already been seen that the higher rate of ACh output maintained in ganglia perfused with plasma must be ascribed to the presence in plasma of a factor (or factors), other than CO_2 , that promotes ACh release. The experiments described in the last paragraph suggested that the plasma factor might be adrenaline. Some experiments on ganglia perfused with eserinated human plasma did not support this idea. Blood was obtained in small quantities (<100 ml) from experienced donors: it was thought unlikely that such blood would contain appreciable amounts of adrenaline. Heparin was used as anticoagulant and the separated plasma was gassed with a $\text{CO}_2\text{-O}_2$ mixture as in the experiments with cat plasma. The plasma choline level is about the same in the two species (18). The results for ACh release and synthesis, when human plasma was perfused through cat ganglia, were indistinguishable from those obtained with cat plasma. It is therefore unlikely that the plasma factor is adrenaline.

A dialyzate of plasma, concentrated so that the diffusible constituents would be present in their original concentration, was eserinated and used as the perfusion fluid in three experiments. Although it was not treated with CO_2 it was as effective in supporting ACh release and synthesis as the plasma from which it was prepared. Further tests showed that the dialyzate retained its activity during 48 hours of storage at 5°C , and in one experiment it lost most or all of its effect when heated to 90° for 10 minutes. It thus appears that the plasma factor (or factors) must be diffusible through cellophane and heat-labile.

Speculation as to its identity would hardly be profitable.

(G) *Plasma from Patients with Myasthenia Gravis*

Since human plasma was found to be a satisfactory perfusion fluid for cat ganglia, the opportunity was taken, with the kind cooperation of Dr. Reuben Rabinovitch, to see whether or not the plasma of patients with severe myasthenia gravis would support ACh release and synthesis equally well. This was found to be the case with three plasma samples which had been stored overnight before being tested. The experiments thus show that myasthenic plasma is not deficient in plasma factor, since this is not lost during overnight storage, nor does it contain a stable inhibitor of ACh synthesis that can act like HC-3 on ganglia. The choline content of one plasma sample was also tested and found to be within the normal range. The possibility that a labile or a slowly acting inhibitor of synthesis might be present in myasthenic blood or muscle (cf. 41) has not been formally excluded.

4. TIME COURSE OF ACh RELEASE DURING REPETITIVE STIMULATION

(A) *ACh Release during Prolonged Stimulation*

Earlier workers have suggested that when a ganglion is stimulated for a long time, ACh is released from a depot of preformed ACh, and that when this depot is depleted by stimulation the final steady efflux is equal to the rate at which the depot can be restocked, either by synthesis (1, 4) or by transformation of synthesized ACh into an available form (5). Our present results are in conformity with this general scheme. They show in addition (a) that most of the ganglion's extractable ACh is in the depot available for release, and (b) that the rate at which the depot can be depleted, or replenished, depends on the composition of the perfusion fluid. We have thought it worth while to see how far the observed facts can be fitted by a simple formula in which the variables are the size of the ACh depot and the rates of ACh release and synthesis.

We shall make the simplifying assumptions that the rate of ACh output at any moment during the stimulation period is proportional to the amount of ACh in the depot, and that ACh is synthesized at a constant rate throughout the period. If these assumptions are correct, then whenever the rate of release exceeds the rate of synthesis the depot ACh must decay exponentially toward a final value that is proportional to the rate of synthesis. For if Y is the depot ACh in μg , which is being released at the fractional rate b/minute , and s is the rate of synthesis in $\mu\text{g}/\text{minute}$, then in any short time dt minutes, $dY/dt = -bY + s$. On integration this gives the amount of ACh in the depot after t minutes of stimulation as

$$[1] \quad Y_t = \frac{C_b}{b} e^{-bt} + \frac{s}{b}$$

where s/b is the final level toward which Y falls, and C_b is constant for any one experiment and is equal to the initial rate of depletion of the depot in $\mu\text{g}/\text{minute}$. The momentary output rate of ACh output at time t minutes is then and

$$[2] \quad Q_t = bY_t = C_b e^{-bt} + s,$$

if the final steady output rate s is subtracted from the successive observed output rates the values of $\log (Q_t - s)$ so obtained should give a linear plot against time.

This hypothesis has been tested with the results shown in Fig. 8, where the ordinates are the mean values of $Q_t - s$ obtained from each of five groups of experiments (Table II, A, B, C, E, G) in which the perfusion fluid did not adequately support ACh synthesis. It was assumed that the asymptotic output rate s , which was not quite reached in an hour's stimulation, would have been 0.5 mμg/minute below the mean output rate for the last 15-minute sample. The values for the last 15 minutes were not plotted since they formed the basis for calculating s . The plotted points show some scatter about their means: this is not surprising in view of the uncertainties introduced by variation between ganglia, assay error, and the varying deficiency of the perfusion fluids in the factors that support ACh release. Nevertheless, most of the points are fitted tolerably well by a straight line. The points for the first 4-5 minutes are an exception, lying on a curve of significantly steeper slope. The relationship is shown more clearly in Fig. 9, in which the over-all mean values of $Q_t - s$ are plotted for each time interval. With the addition of another exponential term to take account of the steeper decay in the first few minutes, equation [2] becomes

$$[3] \quad Q_t = C_a e^{-at} + C_b e^{-bt} + s.$$

The continuous line of Fig. 9 represents equation [3] with fitted values for the constants: 28 and 14 mμg/minute for C_a and C_b , and 0.8 and 0.075 for the rate constants a and b . In the experiments with Locke and HC-3, s may be taken as zero. The output Q_0 at the start of stimulation must then be derived wholly from the original depot, which according to equation [3] consists of two portions: a smaller one $D_a (= C_a/a)$ amounting to 35 mμg, which is more readily released by stimulation and therefore tends to be soon depleted; and a larger one $D_b (= C_b/b)$ amounting to 187 mμg. The depot ACh would thus amount to 222 mμg and the total extractable ACh of the ganglion to 262 mμg if one adds the 40 mμg of 'stationary ACh' found experimentally (cf. 2(B), (b)) not to be available for release. This calculated value for the resting ACh content agrees, as it should, with the observed mean for the control ganglia, which was 266 mμg. From equation [3] it also follows that the final ACh content of a ganglion, in which the depot ACh suffers depletion during 60 minutes of stimulation, should be $(Q_{60}/b - 40)$ mμg, the 40 mμg being the stationary ACh as before. (Q_{60} was not directly measured but could only be a little, say 0.2 mμg/minute, less than the mean output for the last 15 minutes as given in Table II.) The expected final content of the ganglion in each group of such experiments has been calculated from this formula. The values in mμg, with the corresponding observed values in parentheses, are as follows: O₂-Locke, 95 (111); Locke + HC-3, 49 (48); CO₂-Locke, 134 (170); O₂-Locke with high eserine, 71 (166); plasma + HC-3, 61 (61). The agreement in most cases is as good as could be

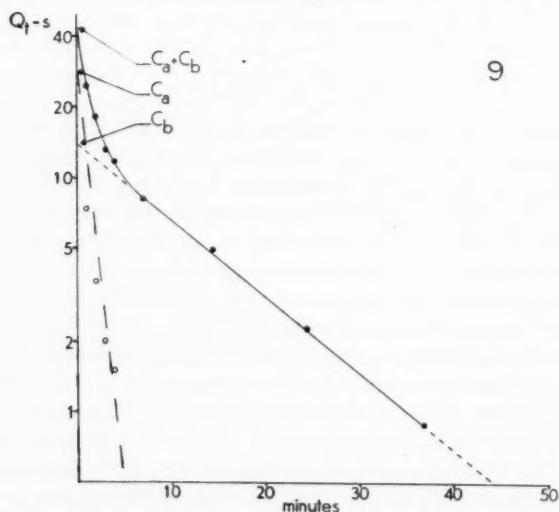
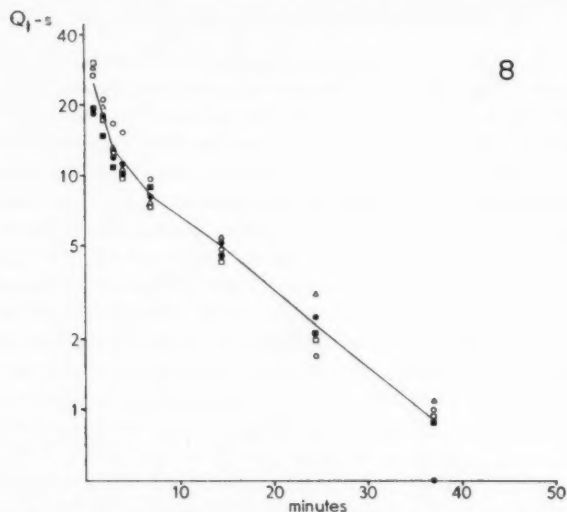


FIG. 8. Time course of ACh release: combined data for all experiments in which depot ACh was depleted by stimulation. $Q_t - s$ is output rate minus final output rate. \circ , O_r -Locke; Δ , O_r -Locke with raised eserine; \square , O_r -Locke + HC-3; \bullet , CO_r - O_r -Locke; \blacksquare , O_r -plasma + HC-3. For explanation see text.

FIG. 9. Time course of ACh release: combined data for all experiments. For explanation see text.

expected, but the tendency of the calculated values to lie below the observed ones suggests that some surplus ACh was still present at the end of stimulation in many cases, especially when the eserine level was high. To a first approxima-

tion, then, the behavior of depot ACh in these depletion experiments conformed satisfactorily with equation [3]. (It should be noted, however, that the ratios C_a/a and C_b/b give a measure of the two portions of the depot only if these release ACh independently of each other. It is, however, possible that one portion is derived from the other, and in that case the respective sizes of the two portions would be different from the figures of 35 and 187 μg given above. This possibility will be discussed later.)

In the experiments in which the ACh depot was kept well stocked throughout the stimulation, because the perfusion fluid was one that supported ACh synthesis, the output declined only during the first 5 minutes and then remained high and steady. Such a finding would be expected if the formation of D_a , the more readily releasable fraction, could not keep pace with the demand although the larger fraction D_b was being well maintained by synthesis. Under these conditions C_b of equation [3] becomes zero and the time course of ACh output should be given by

$$[4] \quad Q_t = C_a e^{-at} + s$$

with C_a and a having their former values. The expected time course of $Q_t - s$ is shown by the steep broken line at the left of Fig. 9. It agrees well with the plotted points representing the mean values of $Q_t - s$ for all those experiments (perfusion with plasma or with choline-Locke, Table II, D, F, H) in which ACh synthesis was adequate, s in each case being taken as the mean output during the last 50 minutes of stimulation. The final steady rate of release in these experiments, according to theory, should be $s = bD_b$, or 14 $\mu\text{g}/\text{minute}$ if $b = 0.075$ as before and D_b is maintained at its maximum value of 187 μg . The observed values of s were 13 $\mu\text{g}/\text{minute}$ for CO_2 -choline-Locke (Table II, D) and 12 $\mu\text{g}/\text{minute}$ for O_2 plasma (Table II, F). The above value for b , however, was derived from experiments in which the perfusion fluid was deficient in one or other of the constituents, CO_2 and plasma factor, necessary for optimal ACh release. Under the more physiological conditions of perfusion with CO_2 -plasma, depot ACh was more readily liberated, and the observed steady-state output was 28 $\mu\text{g}/\text{minute}$, corresponding to a value of 0.15 for b . Too few data were obtained to decide whether a , the rate constant for depletion of the readily releasable fraction D_a , was also higher when this fluid was used for perfusion.

The time course of ACh output during prolonged stimulation at 20/second can now be seen to depend, to a first approximation, on four variables. The first of these is the amount of depot ACh in the ganglion. It varies considerably from animal to animal but averages about 230 μg , or 85% of the total extractable ACh. In a resting ganglion the depot ACh is composed of two fractions of which one, the smaller, contains ACh in a more readily releasable form than the other. The other variables depend on the composition of the perfusion fluid. One of these is the rate at which ACh can be synthesized: this is a limiting factor for ACh output if the perfusion fluid is deficient in choline, or if it con-

tains an inhibitor of ACh synthesis such as HC-3; in earlier work (4) the rate of synthesis was found to depend also on the extracellular glucose level. The remaining two variables are the rates at which ACh can be released by stimulation from either fraction of the depot. The rate of ACh discharge from the smaller fraction was not obviously affected by changes in the composition of the perfusion fluid in our experiments, but from earlier work (26, 27) it is known to depend on the extracellular levels of calcium and magnesium. The rate of ACh discharge from the larger fraction—or alternatively, the rate of ACh transfer from the larger to the smaller fraction—has been shown to depend on the extracellular levels of CO_2 and an unidentified plasma factor, and may also be sensitive to the levels of calcium and magnesium.

The quantity of ACh released by each maximal volley may be calculated from the data discussed above. With the ganglion in its most nearly physiological state (perfusion with plasma equilibrated with $\text{CO}_2\text{-O}_2$) the initial volley output, as determined by extrapolation to zero time, averaged $35\text{ }\mu\text{g}$; the steady-state volley output established after 5 minutes of stimulation averaged $23\text{ }\mu\text{g}$. These figures were derived from experiments in which the preganglionic trunk was stimulated at 20/second. Whether the volley output is altered by changing the frequency of stimulation was investigated in the experiments described below.

(B) ACh Release as a Function of Stimulation Frequency

(a) Plasma Perfusion

In the four experiments whose results are shown in Table III the frequency of stimulation was systematically varied. In each case the perfusion fluid was cat plasma equilibrated with a $\text{CO}_2\text{-O}_2$ mixture to bring its pH close to 7.4, and the preganglionic trunk was stimulated maximally at seven frequencies, 1, 2, 4, 8, 16, 32, and 64/second, taken in random order with 500 volleys at each trial and with 12 minutes of rest between trials. Samples of effluent were collected during and for 2 minutes after each series of volleys, and were assayed against matching dilutions of ACh made up in the perfusion fluid. A small correction, $0.3\text{ m}\mu\text{g}/\text{minute}$, was subtracted from each measured output to allow for the ACh released from the ganglion during rest. The four experiments gave similar results. The volley output was nearly the same at every frequency, and its mean value, $35\text{ }\mu\text{g}$, was the same as the initial volley output (determined by extrapolation) in the experiments with ganglia stimulated for an hour at 20/second. Under these conditions, liberated ACh comes mainly from the smaller and more readily releasable fraction of the depot, and the figure of $35\text{ }\mu\text{g}$ may be regarded as a fair estimate of the volley output from a ganglion in which this fraction has not been depleted by a prior stimulation.

Such a level of volley output cannot be maintained indefinitely in a repetitively stimulated ganglion. Table IV presents the results of three experiments in which stimuli of varying frequency were applied for a longer time. In each experiment the perfusion fluid was plasma equilibrated with CO_2 and O_2 as

before, and the preganglionic trunk was stimulated maximally at 4, 16, and 64/second for 20 minutes with 30 minutes of rest between each period of stimulation. The ACh output in these trials was found to fall off as usual

TABLE III
Release of ACh from plasma-perfused ganglia:
brief stimulation

Volleys/second	Volley output, $\mu\mu\text{g}$				Mean
	1	2	3	4	
1	32	29	52	44	39
2	17	28	47	28	30
4	26	24	52	32	34
8	30	30	52	32	36
16	23	34	47	34	34
32	30	34	49	38	38
64	23	28	54	38	36

TABLE IV
Release of ACh from plasma-perfused ganglia: prolonged stimulation

Volleys/second	Volley output, $\mu\mu\text{g}$				Minute output, $\mu\mu\text{g}$			
	1	2	3*	Mean	1	2	3*	Mean
4	22	37	19	26	5.3	9	4.6	6.3
16	24	37	19	27	23	36	19	26
64	5.5	9.6	5.4	6.8	22	37	21	27

*Choline added.

during the first 5 minutes, but it remained practically steady while the last three 5-minute samples were being obtained. The mean output per volley and per minute for each of these 15-minute periods of steady discharge are given in the table. It can be seen that in every experiment the volley output was about the same at 16/second as at 4/second, but was only one-fourth as great at 64/second. On the other hand, the minute output was about the same at 16/second and 64/second but was only one-fourth as great at 4/second. It follows from the combined results that under these conditions of perfusion, when the readily releasable ACh had been depleted by prior stimulation, the volley output was independent of frequency of stimulation for frequencies up to 16/second and with more rapid stimulation fell off in inverse ratio to the frequency. The maximum volley output for an average ganglion under these conditions was about 27 $\mu\mu\text{g}$; the maximum minute output, in agreement with figures presented earlier (Table II, H), was about 27 $\mu\mu\text{g}$. In one of the experiments of this series the choline content of the perfused plasma had been raised about 100-fold, to $7 \times 10^{-4} M$, by the addition of choline chloride. Since this enrichment with choline did not increase the rate of ACh output, the extracellular choline level cannot be a rate-limiting factor for ganglia perfused with plasma.

(b) *Locke Perfusion*

Perry (5) has shown that the time course of ACh release during prolonged excitation of a Locke-perfused ganglion is substantially independent of stimulation frequency over the range 5–100/second and amounts to about 4 m μ g/minute. We have not thought it necessary to duplicate his observations, but in two experiments we tested the relationship between stimulation frequency and ACh output for brief periods of stimulation in ganglia perfused with O₂-Locke. As in the corresponding trials with plasma perfusion, bursts of 500 volleys were fired down the preganglionic trunk at seven different frequencies over the range 1–64/second. The results differed from those with plasma-perfused ganglia in that the volley output was somewhat higher for the medium frequencies than for the higher and lower ones. It does not seem profitable to speculate on the reasons for the shape of the frequency-output curve under these unphysiological conditions. No evidence was obtained in these experiments, or in those with plasma perfusion, that the volley output at low frequencies or at the outset of stimulation may approach or exceed 100 μ g as has been reported by several authors (19, 27, 42, 43). It is of course probable that the first few volleys of a series may discharge more ACh than the later ones. Indeed, electrophysiological studies strongly suggest that this happens at neuromuscular junctions (44, 45). The possibility cannot, however, be verified by direct measurement of the released ACh, since the quantity involved would be below the threshold for assay.

Discussion

We have confirmed the finding of Brown and Feldberg (1) that a Locke-perfused ganglion stimulated for a long time through its preganglionic trunk releases ACh at a rate that falls off to reach a steady low level. But unlike them we have found that this fall in ACh output is accompanied by an important depletion in the ganglion's content of extractable ACh. The discrepancy between their results and ours on this point is probably to be ascribed to differences in technique. Unless special care is taken to continue maximal stimulation until the moment when the ganglion is removed and placed in the extracting medium, and to prevent any reflux of blood into the ganglion (through vessels accompanying the postganglionic trunk) when perfusion is shut off, it is easy to obtain misleadingly high values for the final ACh content. Some of the data recorded by Brown and Feldberg (1) (and also by Kahlson and MacIntosh (4), who did some similar tests) suggest that these sources of error may have been present in their experiments. Both pairs of workers did, indeed, obtain some evidence for a decrease of ganglionic ACh as a result of prolonged stimulation when the conditions of perfusion were similar to our own: this happened in two of three experiments by Brown and Feldberg (the third experiment gave the opposite result), and in all three experiments by Kahlson and MacIntosh. The mean fall in ACh in these five experiments was 27%, as against 53% in five experiments by us.

Even in our own experiments the observed decrease in ACh content appears at first sight too small to account for the decline in ACh output, averaging over 80%, found in our Locke-perfusion experiments. The explanation for this finding is that even in a normal ganglion a proportion of the ACh is not releasable by nerve impulses, and in a ganglion whose cholinesterase has been inactivated the proportion is higher. The nature of unreleasable ACh is discussed below.

Storage of ACh

We have designated as 'stationary ACh' that fraction of the total store that remains in a ganglion whose ACh has been maximally depleted by prolonged stimulation in the presence of the drug HC-3, which blocks ACh synthesis. It amounts to about 40 μg , or 15% of the total extractable ACh, and apparently can only be set free by procedures that destroy the structural integrity of the ganglion. We have argued that stationary ACh is for the most part located in the extrasynaptic portions of the preganglionic axons; if so, it is presumably separated spatially from the cholinesterase which is also present in these axons. It is attractive to suppose that this fraction is situated in subcellular particles of some kind, in association with the choline acetylase by which it was formed. From the experiments of Hebb, Krause, and Silver (46) on ventral root homogenates it can be concluded that axonal choline acetylase is located in subcellular particles, but whether the same particles contain the axonal ACh is not known. In homogenates of brain, most of the enzyme and most of the ACh sediment together (47), but such homogenates, as Gray and Whittaker (48) have shown, contain intact nerve endings. Their plausible suggestion, that both substances are located in a single kind of particle within these endings has therefore, as they point out, still to be proved. It seems not unlikely, then, that both stationary ACh and releasable ACh are contained within specific subcellular particles, but, as yet, one cannot confidently identify such particles with any of the structures that have been recognized in axons or axonal terminals.

In a ganglion whose cholinesterase remains active the whole of the ACh not included in the stationary fraction is available for release by nerve impulses, as is shown by its disappearance from active ganglia in which synthesis has been prevented by HC-3. This fraction has been denoted 'depot ACh'. It must be located in the nerve terminals, since the preganglionic axons are not depleted of their ACh by stimulation in the presence of HC-3. In an average ganglion the depot ACh amounts to 220 μg , or about 85% of the extractable total. Our analysis of the time course of ACh discharge during prolonged stimulation has shown that depot ACh is composed of two subfractions, one of which is smaller and more readily liberated than the other. In that analysis we assumed, for simplicity, that the two subfractions responded independently to stimulation, each releasing a characteristic proportion of its ACh in response to each arriving volley until the smaller fraction was exhausted. Another

interpretation is, however, equally possible and in some ways more attractive. This is to suppose that the two reservoirs of depot ACh are connected, as it were, in series rather than in parallel, and that ACh from the larger one must pass into the smaller before it can finally be discharged. The two hypotheses account equally well for the time course of ACh release during prolonged stimulation; they also account equally well for the high and constant volley output found when a ganglion that has been rested is briefly stimulated at any frequency over the range 1-64/second, and for the somewhat lower, though still constant, volley output during prolonged stimulation at any frequency up to 16/second. One set of results, however, appears to favor the "series" as against the "parallel" hypothesis. With prolonged stimulation at frequencies above 16/second, the volley output falls off in inverse ratio to the frequency. This fall is not due to depletion of the main ACh depot, for extraction of the ganglion reveals no such depletion; nor can it be due to a progressive failure of some part of the release mechanism, for the volley output, though low, can be maintained indefinitely without further reduction. At the higher frequencies it is the minute output, rather than the volley output, that is independent of frequency; and there must therefore be some process, independent of both the speed of ACh synthesis and the spacing of the incoming volleys, that limits the rate at which ACh can be discharged. This process is most simply imagined, in terms of the "series" hypothesis, as the movement of depot ACh from the larger into the smaller, more readily releasable fraction; in terms of the "parallel" hypothesis one would have to postulate either a further subdivision of the larger fraction, or else that the effectiveness of closely spaced impulses in releasing ACh declines as a precise inverse function of their frequency. Further work, however, will be needed before one hypothesis can be strongly preferred to the other.

It may be noted here that the "series" hypothesis is similar to one proposed by Perry (5), to account for the paradox of a declining ACh output from Locke-perfused ganglia whose ACh stores were supposedly well maintained. Perry suggested, in explanation, that newly synthesized depot ACh could only be made "available" at the rate of about 4 $\mu\text{g}/\text{minute}$. On the basis of our own experiments it seems clear, however, that ACh release under the conditions of his tests must have been limited by the speed of synthesis, rather than by the speed with which synthesized ACh could be made available for release.

In recent years it has been strongly argued that the minute vesicles which abound in the presynaptic axoplasm represent the transmitter depot. Our findings are obviously compatible with this idea. It has been observed with several kinds of synapse (49, 50, 51) that vesicles are not uniformly distributed within nerve endings but show some tendency to be grouped close to the presynaptic membrane. The vesicles so located might be thought to contain the readily releasable fraction of the depot ACh.

Besides stationary and depot ACh, we have described a third kind of intracellular ACh, which is present only in ganglia whose cholinesterase has been

inactivated. This we have called "surplus" ACh. It is formed rather slowly, but may rise to a level above that of the depot ACh. Since it quickly disappears when the enzyme is reactivated, it must be located in a compartment where it would have been destroyed in the presence of the active enzyme. It cannot make an important contribution to the ACh released by stimulation, since the volley output from an eserinated ganglion remains constant while surplus ACh is accumulating. We have pointed out (52) that the formation of surplus ACh, and also the steady release of ACh in minute quantity from the eserinated resting ganglion, are evidence that depot ACh undergoes a continuous slow turnover even when no nerve impulses are arriving at the terminals. It is plausible to suppose that the continuous release during rest may represent a quantal discharge of depot ACh into the extracellular space, such as is known to occur at motor nerve endings in striated muscle, while the continuous formation of surplus ACh may represent a concurrent discharge of depot ACh into the presynaptic axoplasm. But other explanations for these phenomena are conceivable, and it is even possible that surplus ACh is held in structures other than the nerve endings.

Whittaker (53) has recently demonstrated the existence of two forms of bound ACh in a particular subfraction of homogenized brain: one form is readily, one much less readily, set free by simple physical procedures. Since this fraction contained many intact nerve endings (48), and was eserinated, it seems quite likely that the more labile form represented our surplus ACh.

Synthesis of ACh

Although we have confirmed the finding of earlier workers (1, 4) that Locke-perfused ganglia can manufacture important quantities of ACh during stimulation, our experiments emphasize that ACh synthesis in such ganglia is much less efficient than under physiological conditions. The abnormality of synthesis can be completely corrected by adding choline to the Locke; when this is done the ACh turnover of a ganglion can be maintained at a high level as long as stimulation is continued. Normal plasma contains enough choline (about 7×10^{-7} g/ml) to support ACh turnover at the maximum level. A simple calculation shows that the nerve endings must be remarkably efficient in extracting choline from the extracellular fluid. The superior cervical ganglion preparation of the cat when perfused with plasma at our usual rate of 0.3 ml/minute will continue to release ACh at the rate of about 28 $\mu\text{g}/\text{minute}$ during an indefinitely long period of preganglionic stimulation. This ACh must be derived from the plasma choline, which is therefore esterified at the rate of about 21 $\mu\text{g}/\text{minute}$. About half the perfusion fluid flows through structures adjacent to the ganglion, which, if the plasma choline is 700 $\mu\text{g}/\text{ml}$ (18), is supplied with choline at the rate of 100 $\mu\text{g}/\text{minute}$. The nerve endings are therefore able to take up and acetylate some 20% of the choline supplied to the ganglion during the few seconds required for the plasma to pass through the ganglionic vessels. Since choline as a quaternary base diffuses slowly into most

cells, and since the nerve endings can form only a small part of the bulk of the ganglion, this fact is rather remarkable. It suggests that the endings (or perhaps the teloglia elements that embrace them) must be provided with some special mechanism for the entry of choline ions.

We have already (7, 8, 52) recapitulated the evidence that the remarkably specific and potent action of the base HC-3 as an inhibitor of ACh synthesis is to be ascribed to its ability to compete with choline for transport by just such a mechanism. It seems very likely that some sort of choline carrier, located in a membrane lying between the extracellular fluid and the sites of ACh formation, is a constant feature of cholinergic mechanisms. Most of the effects that have been described for HC-3 (6, 12, 54, 55, 56) in the whole animal can be attributed with some confidence to its ability to prevent the synthesis of ACh at cholinergic nerve endings. Blockade of transmission in cholinergic pathways occurs only in the presence of repetitive activity in such pathways, and after an appreciable latency; it is intensified by increasing the frequency of stimulation and it is antagonized by choline. The present paper provides some illustrative examples in the case of the pathway through the superior cervical ganglion: a comparison of the time course of transmission failure with that of ACh release suggests that the volley output must be reduced by about 80% for transmission to be blocked by 50%.

Other bases besides HC-3 can depress ACh synthesis; and we have confirmed earlier reports (35) that eserine in high dosage has such an effect: its mode of action is not necessarily identical with that of HC-3. The possibility that a failure of ACh synthesis at the neuromuscular junction is the basic defect in myasthenia gravis has been raised by Desmedt (45), and prompted us to examine the ability of myasthenic plasma to support synthesis in perfused ganglia. No evidence was found that a circulating inhibitor was present, but either a labile or a slowly acting material might have escaped detection.

Release of ACh

Previous work has emphasized the necessity of external calcium (26, 27, 57) for the release of ACh by the nerve impulse and the antagonistic action of magnesium (27, 57). The concentrations of these ions have not been deliberately varied in our experiments, but we have shown that two other factors must be present in the external fluid for optimal ACh release, even in the case of ganglia whose depot ACh is well maintained. These factors are dissolved CO_2 and an unknown material present in plasma. We have suggested that CO_2 may act by promoting the ionization of calcium, and it is possible that the plasma factor may also be concerned in some way with the uptake or action of calcium.

The amount of ACh released by any series of preganglionic volleys will depend not only on the concentrations of the factors listed above, but also on the amount of depot ACh present in the ganglion at the time and on its partition between the two fractions we have identified. In terms of the "series" hypo-

thesis for which we have expressed a preference, namely that the readily releasable fraction is the immediate source of all released ACh, this fraction in a normal resting ganglion will contain about 50 μg of ACh and there will be about 170 μg in the remainder of the depot. If the extracellular factors for release are maintained at the physiological level, each arriving volley will discharge about 1/1200 of the ACh in the smaller fraction: the proportion discharged will be the same, to a first approximation, whatever the frequency of stimulation, and whether or not the readily releasable fraction has undergone depletion. The ACh lost from that fraction will be replenished from the larger fraction, but the rate of replenishment cannot exceed 28 $\mu\text{g}/\text{minute}$, and will be proportionately smaller if the larger fraction has been depleted or if the smaller fraction has undergone only a small depletion. The transfer of ACh from the larger to the smaller fraction may correspond to a movement of depot ACh towards the synaptic membrane. The larger fraction, in its turn, will be replenished at a rate determined by the conditions for ACh synthesis. (On the basis of the "parallel" hypothesis, the proportion of depot ACh in the readily releasable fraction of a resting ganglion will be somewhat smaller than on the basis of the "series" hypothesis, and that fraction will be reduced to a low level by a few minutes of high-frequency stimulation in the way suggested by equation [3] of Section 4(A).) It should be re-emphasized that the volley output from a ganglion remains high at physiological frequencies of excitation, however long the excitation may last. Thus it may be doubted whether failure of transmission, at unpoisoned cholinergic junctions, is ever due to failure of ACh liberation. If prolonged high-frequency stimulation leads to junctional block, the block must be due to lowered sensitivity of the postsynaptic structures to ACh, or to an excess of free ACh, or to asynchronous release of ACh, rather than to a reduction of volley output.

Relationship of ACh Synthesis to ACh Release

A ganglion supplied with a fluid that supports ACh synthesis has a considerable turnover of ACh even when it is at rest, as is revealed when its cholinesterase is inactivated. In such a ganglion ACh is manufactured at the rate of about 4 $\mu\text{g}/\text{minute}$ and released into the circulation at about 1/10th that rate. During prolonged stimulation at high frequency the rate of release goes up by a factor of perhaps 70 and the rate of synthesis by a factor of 7, the increase in synthesis being somewhat more than is needed to keep pace with the accelerated release. But even under the most favorable conditions of repetitive activity, the choline acetylase of the ganglion is working at only a fraction of its capacity, as is shown by the ability of the enzyme after it has been extracted from a ganglion to form ACh 4 times as fast (22). Synthesis in the intact ganglion may therefore be limited either by the supply of substrate (choline and acetyl-coenzyme A) or by accumulation of the product, ACh, in the vicinity of the synthesizing enzyme. It is unlikely that choline is a limiting factor for synthesis either in the resting or in the active ganglion, unless a drug like HC-3

is present. When a resting ganglion is eserinizied, its ACh content rises by about 25% in the first 5 minutes even in the presence of HC-3, so that there must have been some choline available for ACh synthesis in the nerve endings; and in an active ganglion perfused with plasma, the ACh output cannot be raised by adding more choline to the plasma. The possibility that acetyl-coenzyme A is a limiting factor cannot be excluded, but it is not obvious how synaptic activity could determine the rate at which it is supplied to the enzyme. A more attractive hypothesis is to suppose that the rate-controlling factor for synthesis is the concentration of ACh in the vicinity of the synthesizing enzyme. We have referred to the possibility that the ACh and choline acetylase of brain are contained within the same subcellular particle, which may be the synaptic vesicle; and it can be calculated (7, 58) that if the vesicles do indeed represent the ACh store the concentration of ACh in the vesicular fluid must be very high, even approaching isotonicity. If newly synthesized ACh remains associated with choline acetylase within a vesicle, further synthesis should be retarded so long as the vesicle remains intact, but would be resumed after ACh has been released by an effective collision between the vesicle and the presynaptic membrane. The formation of surplus ACh, on this hypothesis, would suggest that some ACh can escape from the vesicles into the surrounding axoplasm, where it can accumulate if cholinesterase has been inhibited, this accumulation, in turn, ceasing when the concentration of ACh outside the vesicles approaches that inside.

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